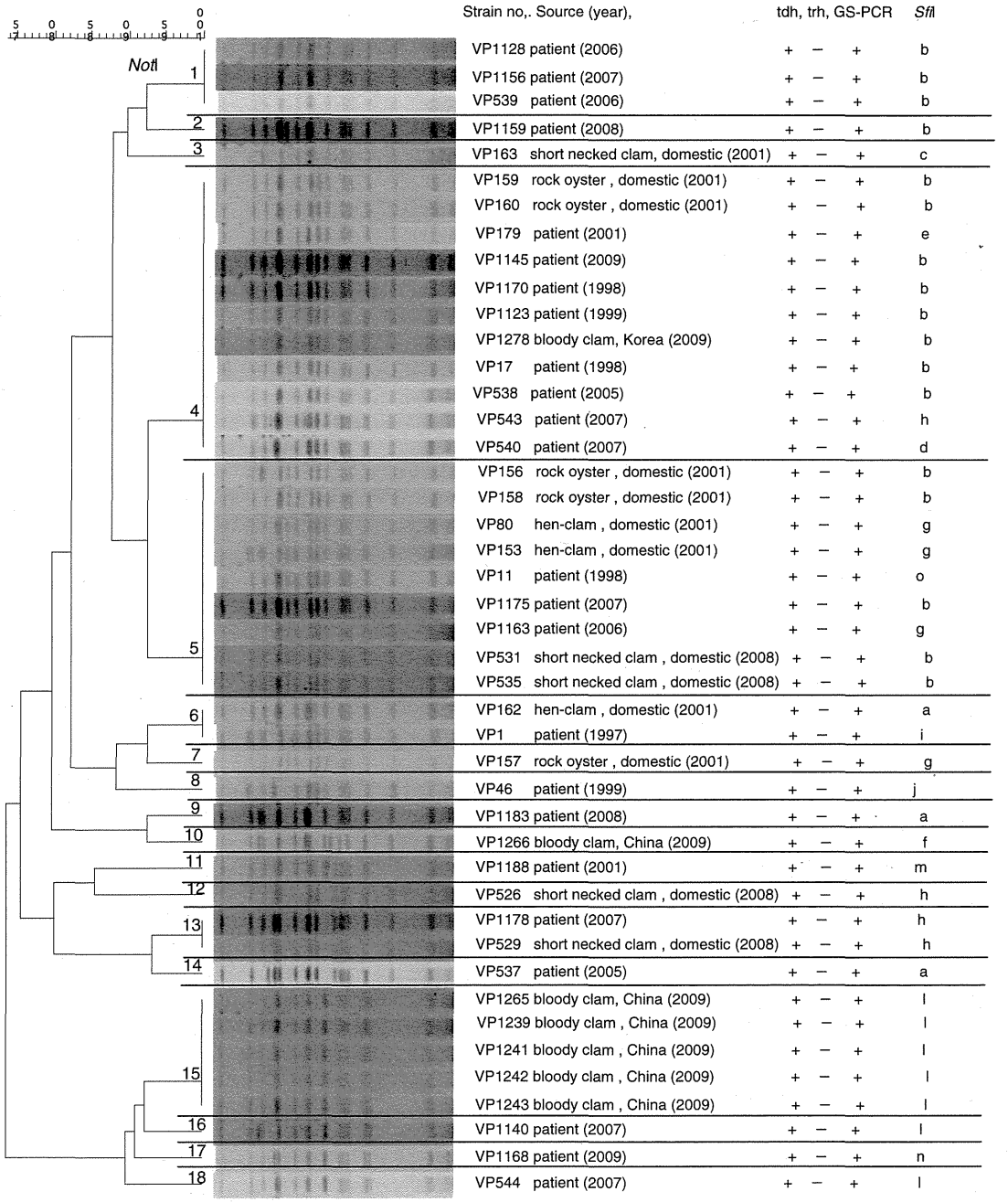

Fig. 5. PFGE profiles of *Not*I-digested genomic DNA of serotype O3:K6 *V. parahaemolyticus* and their relationships.Hundred percent concordances were considered to be indicative of the same profile.

reduction of *V. parahaemolyticus* infections in Japan, the sustained reduction to historic lows in *V. parahaemolyticus* infections caused by all serotypes (Fig. 3) suggests that the hygienic controls on seafood contributed to the reduction in *V. parahaemolyticus* infections in Japan.

We compared the frequencies of total *V. parahaemolyticus*- and *tdh*-positive samples in 2007–2009 with those of our study in 2001 (Hara-Kudo et al., 2003) (Table 1), although *V. parahaemolyticus* infections had been declining since 1999. The decreases of contamination of total *V. parahaemolyticus* and *tdh*-positive *V. parahaemolyticus* were small compared to the decrease in the number of *V. parahaemolyticus* infections; the number of cases and outbreaks decreased 18-fold and 17-fold from 2001 to 2007–2009, respectively.

The frequency of *tdh*-positive *V. parahaemolyticus* in samples exceeding the level was 3 times higher than that in samples below 100 MPN of total *V. parahaemolyticus*/g of seafood. This finding indicates that the standards based on the total *V. parahaemolyticus* levels being below 100 MPN/g of raw-consumption seafood and the total *V. parahaemolyticus*-negative in ready-to-eat boiled seafood may have contributed to the reduction in the consumption of seafood contaminated with *tdh*-positive *V. parahaemolyticus*.

It was suggested that the rapid and large reduction in *V. parahaemolyticus* infections was not due to a change in *V. parahaemolyticus* contamination of seafood at the production and distribution stages. However, improved hygienic conditions for seafood from distribution to consumption may have caused the drastic decrease in the infections. Storage below 10 °C and usage of hygienic seawater or water for seafood, and microbiological standard of total *V. parahaemolyticus* level; negative per 25 g of boiled octopus and crab, and less than 100 MPN/g of seafood for raw consumption in

distribution stage might have contributed to the drastic decrease. Consumption of seafood within 2 h after taking seafood from the refrigerator is also a measure in consumption stage. In a survey with a total of 244 facilities of seafood retail shop or restaurants in a prefecture of Japan, one fourth of them answered that they renewed their kitchens and bought new refrigerators after 2001 (data not shown). In addition, half of them now stored seafood in a refrigerator until immediately before serving. More than half of them made faeces examination of *V. parahaemolyticus* obligatory for employees in seafood retail shop or restaurants.

The other possible factor that could have contributed to the decrease in *V. parahaemolyticus* infections is the temperature of the seawater, which could have affected total *V. parahaemolyticus* level (Blackwell and Oliver, 2008) and the presence of pathogenic *V. parahaemolyticus* (Julie et al., 2010). While there have been some fluctuations in seawater temperatures in coastal Japan, there are no trends of decreasing water temperatures since 1998 (Japan Meteorological Agency, http://www.data.kishou.go.jp/kaiyou/db/kaikyo/series/wnpsst_series1. html). Although seafood consumption decreased from 1998 to 2008 by 13% (The Ministry of Agriculture, Fishery and Forest, 2002, 2009), this decrease is far smaller than the decrease in *V. parahaemolyticus* infections.

In conclusion, the epidemic of *V. parahaemolyticus* infections in Japan is unique due to the sharp decrease in infections by all serotypes, including O3:K6, from 1999 to 2009. Although the pandemic O3:K6 serotype continues to inhabit areas in or around Japan from 1997 to the present, incidences of infections tend to be limited. We demonstrated that the rapid and considerable decrease in *V. parahaemolyticus* infections was not due to changes in *V. parahaemolyticus* contamination of seafood. Rather, a series of governmental control measures implemented in 1999 and 2000 may have facilitated the improvement in hygiene conditions throughout the supply chain, spanning from the distribution to the consumption of seafood and contributing to the reduction in *V. parahaemolyticus* infections in Japan. However, future analysis of the factors related to the decrease in *V. parahaemolyticus* infections should be valuable for the countries in which raw seafood consumption has become preferred and *V. parahaemolyticus* infections have increased (Martinez-Urtazaa et al., 2010; Wang et al., 2007).

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Multiplex PCR assay for identification of three major pathogenic *Vibrio* spp., *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*

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ABSTRACT

A multiplex PCR assay was developed based on *atpA*-sequence diversification for molecular identification of 3 major pathogenic *Vibrio* species: *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. It specifically identified them from among 133 strains of various *Vibrio* species and other genera, and was applicable for testing seawater, suggesting its usefulness.

The genus *Vibrio* is a group of gram-negative bacteria comprising more than 70 species. *Vibrio* species are ubiquitous in aquatic environments and inhabit marine animals as symbionts and commensals. Some of them are pathogenic to animals, including humans [1]. Three species account for a majority of human *Vibrio* infections: *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. Toxigenic (cholera toxin-producing) *V. cholerae* O1/O139 causes cholera, which is an acute intestinal infection caused by ingestion of contaminated food or drink [2]. *V. parahaemolyticus* is a halophilic bacterium that naturally inhabits marine waters and consumption of raw contaminated seafoods causes acute gastroenteritis. In Japan, *V. parahaemolyticus* is the leading cause of *Vibrio* infections [3]. *V. vulnificus* is another pathogenic species that inhabits seawater, and causes gastroenteritis or septicemia. *V. vulnificus* can cause severe infections in immunocompromised and chronic liver disease patients with a high mortality rate [4].

The phenotypic identification of *Vibrio* species is often problematic because there are only a few common characteristics for each species owing to intra-species variation of biochemical characteristics. In addition, such identification is time consuming. The application of molecular methods such as PCR may enable easy and rapid identification of the species. Some rapid detection methods based on PCR have been developed [5–7]. They detect (putative) virulence genes specific for corresponding species encoding such as outer-membrane proteins, hemolysins, and regulatory proteins. To use such virulence genes as identification markers may be of significance because the existence of virulence genes is potentially linked to the pathogenesis. However, when it is applied to investigate environmental samples, there is a potential risk of misidentification, since such genes might transfer among bacteria and create different species with the target genes. Loss of target genes would also occur in environment and also during procedures in testing. Therefore it would be worth designing another PCR method based on the phylogenetic marker. Recently, Thompson et al. performed phylogenetic analysis of vibrios by using the sequences of *atpA*, which is a housekeeping gene that encodes the ATP synthase subunit A, to indicate that *atpA* is a useful phylogenetic marker for vibrios' identification [8]. However, the sequence analysis is needed for identifying *Vibrio* species. Further, it cannot be directly applied to screen an environmental sample, which potentially contains multiple *Vibrio* species. We, therefore, tried to find out unique sequence segments on *atpA* of each pathogenic *Vibrio* species for constructing specific PCR primer sets for the housekeeping gene.

To construct and confirm primer sets for specifically amplification of *atpA* gene of each *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, we compared a total of 199 *atpA* sequences of *Vibrio* species; 74 of which were retrieved from the GenBank database and

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Table 1
Summary of the multiplex PCR assay.

Species	Results of PCR[a]				
	–	vc	vp	vv	n
Aeromonas spp.	4				4
V. alginolyticus	10				10
V. brasliensis	2				2
V. cholerae		25			25
V. diabolicus	4				4
V. fluvialis	1				1
V. furnissii	3				3
V. harveyi	6				6
V. mediterranei	2				2
V. mimicus	5				5
V. natriegens	1				1
V. parahaemolyticus			28		28
V. vulnificus				38	38
Listonella sp.	1				1
Shewanella sp.	1				1
Shigella sp.	1				1
Salmonella sp.	1				1

[a] vc, vp, and vv: identified as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, respectively. –: no amplification products corresponding to the three species.

125 were newly sequenced with *Vibrio* strains representing 12 named species in this study (Table 1, GenBank accession numbers of the sequences: AB597037 to AB597161). These strains were from our laboratory collection; they were from different environments and patients and also included reference strains such as *V. cholerae* NCTC4715 and NCTC4716, *V. parahaemolyticus* RIMD2210633, *V. vulnificus* ATCC27562, *Vibrio mimicus* ATCC33653, *Vibrio fluvialis* ATCC33809, and *Vibrio furnissii* NCTC11328. Finally, the following primers were designed and used as the specific primer sets for multiplex PCR assay system (Fig. 1a): *atpA*- VC–F (5′-AATGGGTC-CATACGCGGAT-3′) and *atpA*-VC-R (5′-TGGTGAAGTyTGTTTTGCACC-3′) for *V. cholerae*, *atpA*-VP-F (5′-TACTAGGCCGCGTAGTA-3′) and *atpA*-VP-R (5′-CGCTGGACGTACACCT-3′) for *V. parahaemolyticus*, and *atpA*-VV-F (5′-ACGGTGCTCTGAAGAACACA-3′) and *atpA*-VV-R (5′-GCAGAAACGTCACCAGCCTGA-3′) for *V. vulnificus*; these yielded

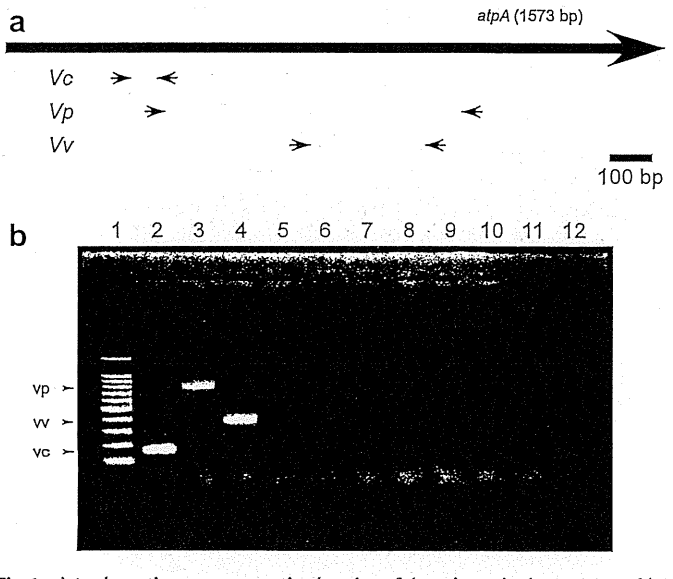

Fig. 1. a) A schematic map representing location of the primers in the *atpA* gene. b) A representative gel image on electrophoresis after the PCR assay. Lane 1, size marker (100-bp ladder); lane 2, *V. cholerae*; lane 3, *V. parahaemolyticus*; lane 4, *V. vulnificus*; lane 5, *V. mimicus*; lane 6, *V. alginolyticus*; lane 7, *V. fluvialis*; lane 8, *Shewanella sp.*; lane 9, *Aeromonas sp.*; lane 10, *Listonella sp.*; lane 11, *Shigella sp.*; and lane 12, *Salmonella sp.* The bands for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are indicated as "vc," "vp," and "vv," respectively, on the left side of the image.

160-bp, 794-bp, and 373-bp amplification products, respectively. PCR was performed using an EmeraldAmp PCR Master Mix (Takara Bio Inc., Shiga, Japan) with 0.5 μM of each primer. The PCR conditions were as follows: initial denaturation at 95 °C for 2 min followed by 25 cycles of denaturation at 95 °C for 20 s, annealing at 65 °C for 20 s, and extension at 72 °C for 1 min. The PCR products were separated on 2% agarose gels, run in 1 × Tris- acetate-EDTA (TAE) buffer, stained with SYBR Safe™ DNA gel stain, and then visualized under a Safe Imager™ blue-light transilluminator (Invitrogen, Carlsbad, CA). Genomic DNA was prepared using a nexttec Genomic DNA Isolation Kit for Bacteria (Toho KK, Tokyo, Japan).

The multiplex PCR assay was carried out with a collection of 133 bacterial strains: 125 *Vibrio* species representing 12 named species; 4 *Aeromonas* species; and 1 strain each of *Listonella*, *Shewanella*, *Shigella*, and *Salmonella* species. The target pathogenic *Vibrio* species were specifically identified based on the specific size of PCR product. PCR product was not amplified from the strains of other *Vibrio* species and of other genera in this assay (Fig. 1b and Table 1). We also examined the strains using *ompW* for *V. cholerae* [7], *toxR* for *V. parahaemolyticus* [6], and *vvhA* for *V. vulnificus* [5]. The results of *ompW* and *toxR* PCR analyses were consistent with those of *atpA*, but 2 of 38 *V. vulnificus* were negative with *vvhA*.

Further, we applied the *atpA*-based PCR assay to conventional culture method for a seawater sample whose enrichment culture was negative for the target species. The sample was diluted and enrichment was performed using alkaline peptone water. After incubating overnight at 37 °C, 1 μl of the enrichment culture were directly subjected to the PCR assay where approximate 1 to 1000 copies of target DNAs were added. As a result, the assay detected as less as 10 copies of the target DNAs either in presence or absence of enrichment culture (Fig. S1).

Vibrio species are naturally diverse bacteria that inhabit aquatic environments. In the environment, they have opportunities exchanging genetic elements such as virulence genes for example chitin-induced transformation. Sometimes organisms lacking genetic markers are created such as *vvhA*-negative *V. vulnificus* described above. Therefore, preferably, probes based on phylogeny in addition to virulence markers should be used. For this purpose the *atpA* gene is one of the candidate, which is a housekeeping gene that can be widely used for phylogenetic analyses and classification [8–11]. The PCR assay described in this study detected target species not only of purified DNA (Fig. 1), but also from colonies on agar plates (data not shown), and of DNA with coexistence of enrichment culture of environmental seawater. The detection limit was as less as 10 copies of DNAs (Fig. S1). This suggests that the PCR assay could detect the target species if there are approximate 10 target cells in 1 μl of enrichment culture. Colony-directed PCR can be applied for rapid isolation of suspicious bacteria, for example, in outbreak investigations. To detect the target pathogens in enrichment cultures such as in the most probable number method can be applied to investigation of environmental and food samples. Thus, we conclude that our assay is a simple but useful and reliable tool for detection of the 3 above-mentioned, major pathogenic *Vibrio* species from human and environmental sources.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.mcp.2011.04.004.

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Molecular Subtyping in Cholera Outbreak, Laos, 2010

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A cholera outbreak in Laos in July 2010 involved 237 cases, including 4 deaths. Molecular subtyping indicated relatedness between the *Vibrio cholerae* isolates in this and in a 2007 outbreak, uncovering a clonal group of *V. cholerae* circulating in the Mekong basin. Our finding suggests the subtyping methods will affect this relatedness.

Cholera is a major public health concern in countries where access to safe water and adequate sanitation cannot be guaranteed for all residents. *Vibrio cholerae* serogroups O1 and O139 are the causative agents of cholera (*1*). A major virulence factor is cholera toxin (Ctx) encoded by the *ctxAB* gene and located on the Ctx prophage. *V. cholerae* O1 is classified into 2 biotypes, classical and El Tor. The El Tor biotype is responsible for the ongoing seventh pandemic of cholera (*2*). Since the early 1990s, the El Tor variant strains, which are biotypes of El Tor but carry the classical type of *ctxB*, have emerged and prevail in multiple regions where cholera is endemic (*1*,*3*–*6*).

The Study

In July 2010, a cholera outbreak began in Attapeu Province in southern Laos along the Cambodian border. Onset dates were July 5–September 16. The outbreak spread to 17 villages of the province and involved 237 persons, including 4 who died. To isolate the suspected *V. cholera* colonies, we screened specimens on thiosulfate citrate bile salt sucrose agar with or without enrichment in alkaline peptone water. Suspected colonies were examined by conventional biochemical tests and PCR amplification of *ctx* (*7*,*8*). Of the 42 fecal specimens tested, 9 were culture positive. The isolates were toxigenic *V. cholerae* O1 serotype Ogawa with features of the El Tor variant, according to the *ctxB*-typing method of Morita et al. (*9*).

We analyzed the 9 *V. cholerae* isolates from the Attapeu outbreak. We performed pulsed-field gel electrophoresis (PFGE) according to the PulseNet protocol (*10*) and multilocus variable number tandem repeat analysis (MLVA) using the 7 loci, as described (*5*,*11*).

The isolates of the Attapeu outbreak had almost indistinguishable PFGE profiles and MLVA repeat copy numbers. In PFGE analysis, 8 of the 9 isolates showed indistinguishable profiles (PFGE-A). The profile of the remaining isolate differed from the dominant isolates by 2 bands (PFGE-B) (Figure). In MLVA, 8 isolates showed the same MLVA type (MLVA-I), and 1 isolate showed another MLVA type that differed from the major MLVA type by being a single-locus variant of MLVA-I with only 1 locus and 1 repeat copy number (MLVA-II) (Table). Seven of the MLVA-I and 1 of the MLVA-II isolates showed the PFGE-A profile, and 1 of the MLVA-I isolates showed the PFGE-B profile. Although the source of contamination remains unknown, these results indicate that all isolates were indistinguishable from or similar to each other and that the outbreak could have been caused by a single source of contamination.

For comparison, we also examined 19 isolates from an outbreak that occurred in Xekong Province in 2007. These isolates also were toxigenic *V. cholerae* O1 serotype Ogawa of the El Tor variant (*12*). MLVA results clearly indicate that the isolates of the Attapeu outbreak in 2010 differed from those of the Xekong outbreak in 2007. The isolates from the Xekong outbreak comprised 3 MLVA types; 17 isolates were MLVA-III, 1 was MLVA-IV, and 1 was MLVA-V. MLVA-IV and MLVA-V were single-locus variants of MLVA-III (Table). Of the 7 loci tested, 3 or 4 displayed different repeat copy numbers than did those of the Attapeu and Xekong outbreaks. In PFGE analysis, however, the profiles were similar to each other; the isolates from the Xekong outbreak showed a PFGE-B profile (Figure).

These results suggest that strains with a specific PFGE type and the related strains have been circulating in the area for at least 3 years. Nguyen et al. suggested that another cholera outbreak in Vietnam that occurred from the end

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Table. MLVA types identified in study of cholera, Laos, 2010*

MLVA type	No. isolates	Outbreak location	*Vibrio cholerae* repeat copy no.							PFGE profile (no. isolates)
			1	2	3	5	6	7	8	
I	8	Attapeu	8	6	NA	NA	7	17	17	A (7), B (1)
II	1	Attapeu	8	6	NA	NA	7	18	17	A (1)
III	17	Xekong	10	6	NA	4	7	16	16	B (17)
IV	1	Xekong	10	6	NA	4	7	16	17	B (1)
V	1	Xekong	9	6	NA	4	7	16	16	B (1)

*MLVA, multilocus variable number tandem repeat analysis; PFGE, pulsed-field gel electrophoresis; NA, no PCR products amplified.

of 2007 to the beginning of 2008 was associated with the Xekong outbreak (*13*). Choi et al. also studied isolates from Vietnam in 2007 and 2008 by using MLVA, wherein they used 5 loci that are in common with those in this study (VC-1, -2, -6, -7, and -8) (*14*). The MLVA results obtained in our study indicated that the repeat copy numbers of the compatible loci of the Xekong outbreak isolates were the same as those of some of the isolates described in the study by Choi et al. This finding strongly suggests that the causative agents of the Xekong outbreak of Laos and the Vietnam outbreak in 2007–2008 were the same. Moreover, the strains were speculated to circulate widely in the Mekong basin, although the similarity between the PFGE profiles of the isolates from Laos and Vietnam remain to be studied.

Recently, another *ctxB* type of *V. cholerae* O1 biotype El Tor serotype Ogawa was reported in Orissa in eastern India (*15*). Representatives of the Xekong and Attapeu isolates also were subjected to sequence analysis of *ctxB*. The results showed that their *ctxB* sequences were identical with that of the original classical type, which suggests that the clonal group in the Mekong basin differs from the new Orissa type of *V. cholerae* in India.

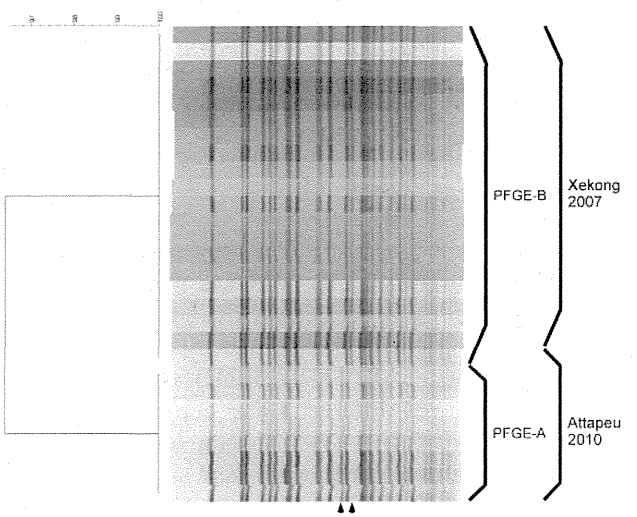

Figure. *Not*I-digested pulsed-field gel electrophoresis (PFGE) profiles of *Vibrio cholerae* isolates, Laos, 2010. The names of the profiles and the sources of the isolates are shown on the right. A dendrogram was created with BioNumerics software (Applied Maths, Kortrijk, Belgium) by using the Dice coefficient, unweighted pair-group method with arithmetic means, and a band-position tolerance of 1.2%. Arrowheads at bottom indicate location of bands differing in PFGE-A and PFGE-B.

Conclusions

Our study clearly indicates that the 2010 cholera outbreak at Attapeu was caused by 1 source of contamination. Furthermore, isolates from the Attapeu outbreak and the 2007 Xekong outbreak showed similar PFGE profiles, but they were differentiated by MLVA, consistent with their origin. This study suggests that PFGE analysis is useful for identifying the kinds of *V. cholerae* clones circulating in a specific geographic region and might be useful for determining a long-term framework of the region-specific *V. cholerae* because PFGE profiles are probably more stable than the MLVA types. By contrast, MLVA is useful for investigating and discriminating short-term individual outbreaks in a region. Another cholera outbreak in Cambodia in 2010 also might be related to the Attapeu outbreak. Combined use of both molecular subtyping methods would indicate the relatedness of cholera in the 2010 Cambodian outbreak and the others in the Mekong basin.

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Identification of a Chitin-Induced Small RNA That Regulates Translation of the *tfoX* Gene, Encoding a Positive Regulator of Natural Competence in *Vibrio cholerae*▽†

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The *tfoX* (also called *sxy*) gene product is the central regulator of DNA uptake in the naturally competent bacteria *Haemophilus influenzae* and *Vibrio cholerae*. However, the mechanisms regulating *tfoX* gene expression in both organisms are poorly understood. Our previous studies revealed that in *V. cholerae*, chitin disaccharide $(GlcNAc)_2$ is needed to activate the transcription and translation of *V. cholerae tfoX* ($tfoX_{VC}$) to induce natural competence. In this study, we screened a multicopy library of *V. cholerae* DNA fragments necessary for translational regulation of $tfoX_{VC}$. A clone carrying the *VC2078-VC2079* intergenic region, designated *tfoR*, increased the expression of a $tfoX_{VC}$::*lacZ* translational fusion constructed in *Escherichia coli*. Using a $tfoX_{VC}$::*lacZ* reporter system in *V. cholerae*, we confirmed that *tfoR* positively regulated $tfoX_{VC}$ expression at the translational level. Deletion of *tfoR* abolished competence for exogenous DNA even when $(GlcNAc)_2$ was provided. The introduction of a plasmid clone carrying the $tfoR^+$ gene into the *tfoR* deletion mutant complemented the competence deficiency. We also found that the *tfoR* gene encodes a 102-nucleotide small RNA (sRNA), which was transcriptionally activated in the presence of $(GlcNAc)_2$. Finally, we showed that this sRNA activated translation from $tfoX_{VC}$ mRNA in a highly purified *in vitro* translation system. Taking these results together, we propose that in the presence of $(GlcNAc)_2$, TfoR sRNA is expressed to activate the translation of $tfoX_{VC}$, which leads to the induction of natural competence.

Natural competence is the ability of bacteria to actively take up DNA from the environment. The imported DNA is homologously integrated into the recipient chromosome to cause a gene exchange, called transformation, while being degraded into nucleotides to be used as a nutrient (15, 34). In most well-characterized naturally competent bacteria (except for constitutively competent bacteria, such as *Neisseria gonorrhoeae* and *Neisseria meningitides* [59]), the development of a competent state is tightly controlled (10), indicating that the advantages of DNA uptake depend on environmental changes.

Cameron et al. proposed that bacteria of the families *Pasteurellaceae*, *Enterobacteriaceae*, and *Vibrionaceae* might share a mechanism to regulate competence genes, whose expression is controlled by two activator proteins, TfoX (also known as Sxy) and CRP (5). Although competence genes are present in these families, only a few species are known to be naturally competent. *Haemophilus influenzae* (*Pasteurellaceae*), one of the well-characterized species, becomes moderately competent as growth becomes slow during late log phase in rich medium and becomes maximally competent when log-phase cells are transferred to a defined starvation medium (23). Under competence-inducing conditions, depletion of sugar imported by a phosphotransferase system causes a rise in cAMP levels, resulting in the formation of its active complex with CRP (cAMP-CRP), which, together with *H. influenzae* TfoX ($TfoX_{HI}$), activates the transcription of competence genes (25, 35, 52). $tfoX_{HI}$ expression is activated at the transcriptional and translational levels in cells in a competence-inducing environment (6, 52). The involvement of the cAMP-CRP complex in transcriptional control has already been suggested (6, 77). Although the $tfoX_{HI}$ mRNA secondary structure plays a crucial role in translational control (6), the regulatory mechanism is not fully understood.

Vibrio cholerae, the agent of cholera, causes a dehydrating diarrheal illness. However, outside the human host, *V. cholerae* lives in natural aquatic environments, such as rivers, estuaries, and coastal waters, where it often associates with chitinaceous zooplanktons, such as copepods (32). Chitin, a polymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc), not only serves as a surface for colonization in aquatic habitats (9, 42, 53) and as a carbon and nitrogen source (24, 30, 42, 49) but also induces natural competence (41). Blokesch and Schoolnik demonstrated that the whole O1-specific antigen gene cluster of *V. cholerae* O1 El Tor can be exchanged by the O37- or O139-antigen gene cluster through chitin-induced natural competence (4). In addition, the cholera toxin prophage (65) and clusters of metabolic genes (43) can be transferred in the same manner. Recently, other *Vibrio* species, *Vibrio vulnificus*, *Vibrio fischeri*, and *Vibrio parahaemolyticus*, were shown to be naturally transformable upon exposure to chitin (8, 17, 50). Thus, chitin-induced natural competence may be a common feature of different *Vibrio* spp. This mode of gene transfer seems to be limited to niches where chitin is abundant in the environment, for example, on the surface of copepods. *Vibrio* species are the

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
Escherichia coli		
DH1	*endA1 gyrA96 hsdR17*(r_K^- m_K^+) *recA1 relA1 supE44 thi-1* F^-	20
SYEC001	DH1 FRT::*cat*::FRT:: bla_P:: $tfoX_{VC(+1\ to\ +709)\text{-}TL}$::*lacZ*	This study
SYEC001S	DH1 FRT::bla_P::$tfoX_{VC(+1\ to\ +709)\text{-}TL}$::*lacZ*	This study
SYEC001SQ1	SYEC001S Δ*hfq*::*kan*	This study
Vibrio cholerae		
V060002	Biovar El Tor	71
SY0603S	V060002 Δ*lacZ*::FRT	71
SY0605	SY0603S *ctxA*::*lacZ*::FRT::*cat*::FRT	71
SY0608S	V060002 Δ$tfoX_{VC}$::FRT	71
SY0626K	V060002 Δ*tfoR*::FRT::*kan*::FRT	This study
SY0612S26K	SY0612S Δ*tfoR*::FRT::*kan*::FRT	This study
SY0613S	SY0603S $tfoX_{VC\text{-}TC}$::*lacZ*::FRT	71
SY0613S26K	SY0613S Δ*tfoR*::FRT::*kan*::FRT	This study
SY0616S	SY0603S $tfoX_{VC\text{-}TL}$::*lacZ*::FRT	71
SY0616S26K	SY0616S Δ*tfoR*::FRT::*kan*::FRT	This study
SY0617	SY0603S FRT::*cat*::FRT::tac_p::$tfoX_{VC(\Delta\text{-}38\ to\ -1)\text{-}TL}$::*lacZ*::FRT	71
SY0620	SY0603S FRT::*cat*::FRT::tac_p::$tfoX_{VC(\Delta-38\ to\ +92)\text{-}TL}$::*lacZ*::FRT	71
SY0627	SY0603S $tfoR_{(+78)}$::*lacZ*::FRT::*cat*::FRT	This study
SY0628	SY0603S $tfoR_{(-30)}$::*lacZ*::FRT::*cat*::FRT	This study
Plasmids		
pBAD33	$araBAD_p$ expression vector, *cat* $araBAD_p$	18
pBAD-SYR1	pBAD33 $tfoR_{(+1\ to\ +102)}$	This study
pCP20	Flp expression plasmid, *bla cat cI*857 λR_P *flp ori* pSC101	12
pGEM-T	TA cloning vector, *bla*	Promega
pKD3	Template plasmid for Red system, *bla* FRT::*cat*::FRT *ori* R6K	12
pKD4	Template plasmid for Red system, *bla* FRT::*kan*::FRT *ori* R6K	12
pKD46	Red expression plasmid, *bla* $araBAD_p$ *gam exo ori* pSC101	12
pRL124	*lacZ* fusion vector, *bla lacZ*	39
pRL-SYX1	pRL124 $tfoX_{VC(-238\ to\ +308)\text{-}TL}$::*lacZ*	This study
pRL-SYX2	pRL124 tac_p::$tfoX_{VC(+1\ to\ +308)\text{-}TL}$::*lacZ*	This study
pRL-SYX3	pRL124 tac_p::$tfoX_{VC(+93\ to\ +308)\text{-}TL}$::*lacZ*	This study
pRL-SYX4	pRL124 tac_p::$tfoX_{VC(+93\ to\ +308)\text{-}TL}$::*lacZ* ($G_{+104}$ to C)	This study
pRL-SYX5	pRL124 tac_p::$tfoX_{VC(+93\ to\ +308)\text{-}TL}$::*lacZ* ($A_{+119}$ to C)	This study
pRL-SYX6	pRL124 tac_p::$tfoX_{VC(+93\ to\ +308)\text{-}TL}$::*lacZ* ($A_{+125}$ to C)	This study
pRL-SYX7	pRL124 tac_p::$tfoX_{VC(+93\ to\ +308)\text{-}TL}$::*lacZ* ($G_{+113}$ G_{+114} to CC)	This study
pRL-SYX8	pRL124 tac_p::$tfoX_{VC(+93\ to\ +308)\text{-}TC}$::*lacZ*	This study
pRL-SYX9	pRL124 tac_p::$tfoX_{VC(+93\ to\ +308)\text{-}TC}$::*lacZ* ($A_{+125}$ to C)	This study
pTH18ks5	Suicide plasmid, *kan ori* pSC101	21
pTrc99A	tac_P expression vector, *bla* tac_P	3
pTrc-hfq	pTrc99A hfq_{EC}	44
pTrc-SYQ1	pTrc99A hfq_{VC}	This study
pUC118	Cloning vector, *bla*	72
pUC-SYR1	pUC118 $tfoR_{(-85\ to\ +247)}$	This study

most abundant bacterial species associated with copepods (55, 57), where there may be a pool of DNA from closely related species. Under such environment, chitin might serve as a signal for these bacteria to sense a DNA source suitable for genetic exchange via transformation.

In *V. cholerae*, $TfoX_{VC}$ (an ortholog of $TfoX_{HI}$) plays a central role in chitin-induced competence (41, 42). The expression of the $tfoX_{VC}$ gene is activated in response to chitin, and $TfoX_{VC}$ upregulates competence genes, including *VC1917*, encoding an essential competence protein homologous to *Bacillus subtilis* ComEA, and structural genes for type IV pilus-like DNA uptake machinery (*pilA*, *pilB*, *pilQ*, etc). $TfoX_{VC}$ also positively regulates several genes (*VCA0027*, *VC1952*, *VC0679*, and *VC0972*) for extracytoplasmic chitin degradation functions (41). Our previous studies demonstrated that chitin, more specifically the disaccharide $(GlcNAc)_2$, activates both the transcription and translation of $tfoX_{VC}$ to induce competence (71). Therefore, it is suggested that *H. influenzae* and *V. cholerae* use a similar mode of genetic control for competence development. In this study, we screened a multicopy library of *V. cholerae* DNA fragments and identified a novel small RNA (sRNA) involved in translational regulation of $tfoX_{VC}$. This is the first report to identify a posttranscriptional regulator of the *tfoX* gene among naturally competent species.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and DNA manipulation. All the strains and plasmids used in this study are listed in Table 1. The *Escherichia coli* strain

JM109 (72) was used for DNA cloning. The bacteria were grown in Luria-Bertani (LB) broth and agar and SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). The antibiotics used and their concentrations were as follows: 100 μg/ml of ampicillin (Ap), 12.5 μg/ml (for *E. coli*) or 1 μg/ml (for *V. cholerae*) chloramphenicol (Cm), and 30 μg/ml kanamycin. Arabinose, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and isopropyl-β-D-thiogalactopyranoside (IPTG) were used at concentrations of 5 mg/ml, 100 μg/ml, and 1 mM, respectively. PCR amplification of DNA was carried out with a GeneAmpPCR system 9700 (Applied Biosystems) by using the Herculase II fusion enzyme (Stratagene). DNA sequencing was performed using a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems). Customized primers, the nucleotide sequences of which are listed in Table S1 in the supplemental material, were purchased from Hokkaido System Sciences. Chromosomal and plasmid DNAs were extracted using a DNeasy blood and tissue kit (Qiagen) and a High Pure plasmid isolation kit (Roche), respectively.

Protein analysis. SDS-PAGE and Western blotting of proteins were performed according to previously described methods (70).

Chromosomal engineering. Genetically modified *E. coli* and *V. cholerae* strains were constructed using the λ Red-FLP recombination system (12, 70, 71). Briefly, the procedure includes 3 processes: (i) preparation of a PCR fragment typically containing 2 Flp recognition target (FRT)-linked antibiotic resistance gene cassettes with flanking regions homologous to the target gene (*X*::FRT::*cat*::FRT::*X′* or *X*::FRT::*kan*::FRT::*X′* fragment, where *X* is the upstream homologous sequence and *X′* is the downstream homologous sequence; the typical size of *X* or *X′* is ~50 bp for *E. coli* or ~1,000 bp for *V. cholerae*); (ii) introduction of the PCR fragment into the recipient strain expressing λ Red recombinase to integrate the exogenous DNA into the chromosome; and (iii) elimination of the antibiotic resistance gene by FLP recombinase if necessary.

(i) Preparation of the PCR fragment to construct the bla_P::$tfoX_{VC}$::*lacZ* translational fusion in *E. coli*. The PCR fragment was generated by a 2-step PCR. pKD3 (12) was used as a template to amplify the FRT::*cat*::FRT cassette flanked by the 50-bp sequence homologous to the upstream region of the *E. coli lac* promoter and the 20-bp sequence homologous to the upstream region of the *bla* promoter (bla_P) using the primers EClacI-FRTf1/FRT-blar1. On the other hand, the bla_P fragment flanked by the 20-bp sequence homologous to $tfoX_{VC}$ (nucleotides +1 to +20) was amplified using the primers FRT-blaf1/bla-tfoXr1 and pKD3. Additionally, we amplified the $tfoX_{VC}$ fragment (nucleotides +1 to +709) flanked by the 50-bp sequence homologous to *lacZ* by using the primers bla-tfoXf1/tfoX-lacZr6 and chromosomal DNA from V060002. These 3 PCR fragments were purified using a High Pure PCR product purification kit (Roche). Then, 0.3 pmol of each of the three PCR fragments was mixed with the primers EClacI-FRTf1/tfoX-lacZr6. The PCR program was as follows: 95°C for 2 min, followed by 25 cycles at 95°C for 30 s, 40°C for 30 s, and 72°C for 2 min, which yielded the DNA fragment *lacZ*::FRT::*cat*::FRT::bla_P::$tfoX_{VC\text{-}TL}$::*lacZ′*.

(ii) Preparation of the PCR fragment for *hfq* gene disruption in *E. coli*. The PCR fragment for *hfq* disruption was prepared as described previously (44). The 500-bp upstream and downstream regions flanking *hfq* were amplified using the primer sets hfqU1/hfqU2 and hfqD1/hfqD2. Additionally, the *kan* cassette was amplified from pTH18ks5 (21) using the primers Km1/Km2. These 3 PCR fragments were purified and then connected by PCR using the primers hfqU1/hfqD2 to obtain the DNA fragment *hfq*::*kan*::*hfq′*.

(iii) Preparation of the PCR fragment for *tfoR* gene disruption in *V. cholerae*. The 2-step PCR protocol described in section i was used to generate the DNA fragment. Chromosomal DNA from V060002 was used as a template to amplify the 1,000-bp upstream and downstream regions flanking *tfoR* with the primer sets TFORf15/TFOR-FRTr1 and TFOR-FRTf1/TFORr1, respectively. Additionally, pKD4 (12) was used as a template to amplify the FRT::*kan*::FRT cassette with the primers FRTf1/FRTr1. These 3 PCR fragments were purified and then connected by PCR using the primers TFORf15/TFORr1 to obtain the DNA fragment *tfoR*::FRT::*kan*::FRT::*tfoR′*.

(iv) Preparation of the PCR fragment to construct the $tfoR_{+78}$::*lacZ* transcriptional fusion in *V. cholerae*. The PCR fragment for construction of the $tfoR_{+78}$::*lacZ* transcriptional fusion was generated by a 2-step PCR. Chromosomal DNA from V060002 was used as a template to amplify the 1,000-bp upstream and downstream regions flanking *tfoR* with the primer sets TFORf15/TFORr10 and TFORf14/TFORr1, respectively. Additionally, we amplified the *lacZ*::FRT::*cat*::FRT fragment with the primers TFOR-LACZf3/TFOR-FRTr3 by using chromosomal DNA from SY0605, which contains a *lacZ*::FRT::*cat*::FRT cassette within the *ctxA* gene. These 3 PCR fragments were purified and then connected by PCR using the primers TFORf15/TFORr1. The PCR program was as follows: 95°C for 2 min, followed by 25 cycles at 95°C for 30 s, 40°C for 30 s, and 72°C for 4 min, which generated the DNA fragment $tfoR_{+78}$::*lacZ*::FRT::*cat*::FRT::*tfoR′*.

(v) Preparation of the PCR fragment to construct the $tfoR_{-30}$::*lacZ* transcriptional fusion in *V. cholerae*. The 2-step PCR protocol described in section iv was used to amplify the DNA fragment. Chromosomal DNA from V060002 was used as a template to amplify the 1,000-bp upstream and downstream regions flanking *tfoR* with the primer sets TFORf15/TFORr13 and TFORf14/TFORr1, respectively. We also amplified the *lacZ*::FRT::*cat*::FRT fragment with the primers TFOR-LACZf5/TFOR-FRTr3 by using chromosomal DNA from SY0605. These 3 PCR fragments were purified and then connected by PCR using the primers TFORf15/TFORr1 to obtain the DNA fragment $tfoR_{-30}$::*lacZ*::FRT::*cat*::FRT::*tfoR′*.

(vi) Recombination by λ Red and FLP. *In vivo* recombination mediated by λ Red and FLP was conducted as described previously (70, 71). The PCR products were purified as mentioned above, ethanol precipitated, and dissolved in 20 μl of 10% glycerol. Each DNA (2 μg) was electroporated into the recipient *E. coli* and *V. cholerae* strains expressing λ Red recombinase. Table S2 in the supplemental material shows the specific combinations of donor DNA and recipient strain for the chromosomal engineering.

Construction of pRL-SYX1. The fragment including the upstream sequence of $tfoX_{VC}$ (nucleotides −238 to +308) was PCR amplified from chromosomal DNA of V060002 using the primers TFOXPf1S/TFOXPr1B and then cloned into the SalI/BamHI site of pRL-124 (39) to generate pRL-SYX1.

Construction of pRL-SYX2, pRL-SYX3, pRL-SYX4, pRL-SYX5, pRL-SYX6, and pRL-SYX7. The fragment including $tfoX_{VC}$ (nucleotides +1 to +308) fused to the *tac* promoter (tac_p::$tfoX_{VC}$) was PCR amplified from chromosomal DNA of SY0617 (71) using the primers PTRCf1S/TFOXPr1B and then cloned into the SalI/BamHI site of pRL-124 to generate pRL-SYX2. Using the same primers, a fragment of tac_p::$tfoX_{VC}$ (nucleotides +93 to +308) was amplified from chromosomal DNA from SY0620 (71) and then inserted into the SalI/BamHI site of pRL-124 to yield pRL-SYX3. The same regions with a single point mutation were amplified by 2-step PCR. Using several primer sets, PTRCf1S/TFOXmtr1 and TFOXmtf1/TFOXPr1B for pRL-SYX4, PTRCf1S/TFOXmtr2 and TFOXmtf2/TFOXPr1B for pRL-SYX5, PTRCf1S/TFOXmtr4 and TFOXmtf4/TFOXPr1B for pRL-SYX6, and PTRCf1S/TFOXmtr18 and TFOXmtf18/TFOXPr1B for pRL-SYX7, the 4 fragment sets were first amplified from pRL-SYX3. These fragments were purified and then fused by PCR using the primers PTRCf1S/TFOXPr1B to be cloned into the SalI/BamRI site of pRL124, generating the respective plasmids (pRL-SYX2, pRL-SYX3, pRL-SYX4, pRL-SYX5, pRL-SYX6, and pRL-SYX7).

Constructions of pRL-SYX8 and pRL-SYX9. The tac_p::$tfoX_{VC}$ fragment (nucleotides +93 to +308) was PCR amplified from pRL-SYX3 using the primers PTRCf1S/TFOXPr1E and then cloned into the SalI/EcoRI site of pRL-124 to generate pRL-SYX8. The same region with a single point mutation was amplified using the same primers and the template pRL-SYX6 and then inserted into the SalI/EcoRI site of pRL-124, yielding pRL-SYX9.

Construction of pBAD-SYR1. The *tfoR* gene was PCR amplified using the primers tfoRf6Sc/tfoRr3H and chromosomal DNA from V060002. The amplified product was digested with SacI and HindIII and inserted into pBAD33 (18) to generate pBAD-SYR1.

Construction of pTrc-SYQ1. The *hfq* gene from *V. cholerae* was PCR amplified using the primers HFQf2E/HFQr2S and chromosomal DNA from V060002. The amplified product was digested with EcoRI and SalI and inserted into pTrc99A (3) to generate pTrc-SYQ1.

Library screening. Chromosomal DNA from V060002 was sonicated and then blunt ended using an End-It DNA end repair kit (Epicentre) according to the manufacturer's instructions. The DNA fragments were cloned into the HincII site of pUC118 (72). Transformations into SYEC001S were performed by electroporation. Cells were plated on LB agar containing Ap and X-gal to produce about 300 colonies per plate and incubated at 37°C. About 20,000 colonies were screened, and a single blue colony was isolated. Plasmid DNA was extracted, and the inserted fragment was sequenced using the primer M13RV.

β-Galactosidase enzyme assay. β-Galactosidase activity was assayed as described previously (71) using *E. coli* and *V. cholerae* cells grown under the following culture conditions with each sample assayed in triplicate.

(i) *E. coli*. Cells were grown to an optical density at 600 nm of ~0.4 in LB at 37°C. If necessary, Cm, Ap, arabinose, or IPTG was added to the medium.

(ii) *V. cholerae*. Cells were grown in M9 minimal medium containing minimal essential medium vitamin solution (Gibco) and 5 mM GlcNAc or $(GlcNAc)_2$ (Seikagaku Biobusiness) as described previously (71). If necessary, Ap was added to the medium.

Natural transformation. Natural transformation was performed using the recipient *V. cholerae* cells grown in M9 minimal medium containing minimal

essential medium and 5 mM GlcNAc or $(GlcNAc)_2$ and chromosomal DNA from SY1001, which harbors a *cat* cassette in the *ctxB* gene, as described previously (71). If necessary, Ap was added to the medium. Transformation efficiency was defined as Cm-resistant (or Cm-and Ap-resistant) CFU divided by total viable (or Ap-resistant) CFU. Experiments were done at least twice.

Synthesis of digoxigenin-labeled RNA probe for Northern blotting. The RNA probe complementary to *tfoR* was prepared as follows. The DNA fragment with the T7 promoter sequence was PCR amplified using the primers T7-tfoRr1/tfoRf3 and chromosomal DNA from V060002. This amplified DNA was used as a template for *in vitro* RNA synthesis from T7 RNA polymerase with a digoxigenin (DIG) RNA labeling kit (Roche). The DIG-labeled RNA was precipitated by ethanol and dissolved in diethylpyrocarbonate-treated H_2O.

Northern blotting. Cells were cultured under the same conditions used in β-galactosidase assays. RNAs were extracted from the cells using Isogen (Wako) according to the manufacturer's instructions. The RNAs (1.5 μg) were heated and cooled quickly; the RNAs were then separated electrophoretically on a 6% polyacrylamide gel containing 6 M urea. RNA Century-plus size markers (Ambion) were also separated as a molecular standard on the same gel. After electrophoresis, RNAs were electrotransferred onto a Hybond-N+ nylon membrane (GE Healthcare). After UV cross-linking, the membrane was treated for 2 h at 65°C in hybridization buffer (DIG Easy Hyb; Roche). Then, the membrane was incubated for 12 h at 65°C in hybridization buffer containing DIG-labeled RNA probe. After hybridization, the membrane was first washed under a low-stringency condition in 2× SSC (1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) containing 0.1% SDS for 10 min at room temperature and then under a high-stringency condition in 0.2× SSC containing 0.1% SDS for 30 min at 65°C. The membrane was treated with maleic acid buffer (0.1 M maleic acid [pH 7.5], 0.15 M NaCl) containing 1% blocking reagent (Roche), incubated for 20 min with 10,000-fold-diluted anti-DIG-AP Fab fragments (Roche), and then washed twice with maleic acid buffer containing 0.3% Tween 20. DIG-labeled signals were amplified by hydrolysis of disodium 3-(4-methoxyspiro{1,2-dioxetane-3.2′-(5′-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4yl)phenylphosphate (Roche) in detection buffer (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl) and visualized on a Hyperfilm ECL film (GE Healthcare).

Circularization RT-PCR. Circularization reverse transcription (RT)-PCR (11, 73) was performed to determine the 5′ and 3′ ends of TfoR sRNA as follows. The total RNA used in Northern blotting [isolated from cells cultured in the presence of $(GlcNAc)_2$] was also used for self-ligation. Prokaryotic mRNA has a triphosphate at its 5′ terminus. To render the 5′ terminus ligatable, the triphosphate was removed by phosphatase and then monophosphate was added to the 5′-OH end by using a KinaseMax kit (Ambion). Then, the processed RNA (8 μg) was subjected to a reaction with T4 RNA ligase (Takara). cDNA was reverse transcribed from 4 μg of ligated RNA with the primer tfoR-CRTr1, using avian myeloblastosis virus (AMV) reverse transcriptase XL (Takara) according to the manufacturer's instructions. The 5′-3′ junction region in the cDNA was PCR amplified using the primers TFORr5/tfoR-CRTf1. The major products obtained (Fig. 3B) were gel purified and cloned into pGEM-T (Promega). Seven clones from independent transformants were sequenced using the primer T7P.

Synthesis of RNAs for *in vitro* translation. DNA templates carrying a T7 promoter sequence were generated by PCR using chromosomal DNA from V060002 and specific primers. For TfoR (nucleotides +1 to +102) and its cRNA, the primer sets T7-tfoRf6/tfoRr3 and T7-tfoRr1/tfoRf17, respectively, were used. For transcription templates generating $tfoX_{VC}$::*flag* RNAs, full-length RNA (nucleotides +1 to +709 translationally fused to the FLAG tag sequence), and ΔTLE RNA (nucleotides +93 to +709 translationally fused to the FLAG tag sequence), we performed a 2-step PCR: the first round of PCR was carried out using the primers T7-TFOXUTRf42/TFOX-2FLAGr1 for full-length RNA and the primers T7-TFOXUTRf6/TFOX-2FLAGr1 for ΔTLE RNA; the second round of PCR was done using the primers T7-TFOXUTRf42/2FLAG-T7Tr1 for full-length RNA and the primers T7-TFOXUTRf6/2FLAG-T7Tr1 for ΔTLE RNA. Using each amplified DNA as the template, RNA was synthesized by T7 RNA polymerase with a MAXIscript T7 kit (Ambion). After the reaction, the template DNA was degraded by Turbo DNase (Ambion) and the synthesized RNA was purified using a MEGAclear kit (Ambion).

***In vitro* translation.** An *in vitro* translation reaction was performed in a highly purified system (PURE system, purchased from New England BioLabs as Purexpress) (54). The translation mixture (25 μl) contained 40 nM $tfoX_{VC}$::*flag* RNA and various concentrations (0, 40, 200, and 400 nM) of TfoR. After incubation for 20 min at 37°C, the synthesized $TfoX_{VC}$::FLAG proteins were detected by Western blotting using a monoclonal anti-FLAG M2-horseradish peroxidase antibody (Sigma).

RNAhybrid analysis. The RNAhybrid software program (26) was run on the website http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html using the sequences of *tfoR* (nucleotides +1 to +102) and $tfoX_{VC}$ (nucleotides +43 to +133).

BLAST analysis. The sequences homologous to *tfoR* were searched using BLAST (1) on the web site http://www.genome.jp/kegg/. The genome sequences used were from *V. cholerae* N16961 (accession no. AE003852.1), *V. parahaemolyticus* 2210633 (accession no. BA000031.2), *V. vulnificus* CMCP6 (accession no. AE016795.3), and *V. fischeri* ES114 (accession no. CP000020.2).

Multiple alignment. The alignment among the *tfoR* homologues was done using CLUSTAL W (62) in the GENETYX-MAC version 12.0.9 software program (Genetyx Corporation).

Nucleotide sequence accession number. The *tfoR* sequence was deposited in GenBank and assigned the accession number AB598400.

RESULTS

Reevaluation of DSE in $tfoX_{VC}$. Previously, we identified 2 *cis*-acting elements for translational control of $tfoX_{VC}$, a translational control element (TLE) nucleotides +43 to +84) and a downstream element (DSE) (nucleotides +107 to +133) (71) (Fig. 1A). TLE negatively controls translation in the absence of $(GlcNAc)_2$, whereas DSE seems to play an essential role in translation. DSE is located downstream of a putative Shine-Dalgarno (SD) sequence (GGGA, +98 to +101) and start codon (GTG, +104 to +106), which are predicted on the basis of the GenBank sequence annotation (accession number AE003852.1), and thereby it has been postulated to be a translational enhancer, such as a downstream box (60, 71). By careful reannotation, however, we found that there is another conservative SD sequence (AGGA, +112 to +115) located with other start codons (ATG, +119 to +121; ATG, +125 to +127) within DSE (Fig. 1A). This suggests a possibility that DSE overlaps the translational start region of $tfoX_{VC}$. To clarify this, we introduced a point mutation in each potential start codon within a $tfoX_{VC}$::*lacZ* translational fusion on the plasmid pRL124. We first constructed a fusion plasmid, pRL-SYX3, in which both the native promoter ($tfoX_{VCp}$) and TLE were exchanged with the *tac* promoter (tac_p) to constitutively drive transcription and translation (Fig. 2). This enabled us to exclude a possibility that point mutation may affect the regulation of $tfoX_{VC}$ expression. Three other plasmids (pRL-SYX4, pRL-SYX5, and pRL-SYX6) (Fig. 2) were constructed from pRL-SYX3. Derivatives of *V. cholerae* strain V060002 containing a fusion plasmid with or without a single base change were tested for β-galactosidase activity when they were grown in M9 minimal medium in the presence of GlcNAc or $(GlcNAc)_2$ (Fig. 2). Consistent with our previous data using chromosomal fusion (71), the addition of $(GlcNAc)_2$ increased β-galactosidase activity from strains carrying pRL-SYX1 (15-fold) and pRL-SYX2 (21-fold), which contain the wild-type leader sequence situated downstream of $tfoX_{VCp}$ and tac_p, respectively, compared with that of GlcNAc, while the strain carrying pRL-SYX3 had a high activity of β-galactosidase under both culture conditions (Fig. 2). A similar pattern of expression was observed in strains carrying pRL-SYX4 and pRL-SYX5, which have base changes of GTG at positions +104 to +106 ($\underline{G}TG_{+104\ to\ +106}$) to CTG and $\underline{A}TG_{+119\ to\ +121}$ to CTG, respectively (the underlined nucleotide was changed), indicating that these nucleotides are not essential for translation. On the other hand, β-galactosidase activity in the strain with pRL-SYX6, in which $\underline{A}TG_{+125\ to\ +127}$ was replaced by CTG, was

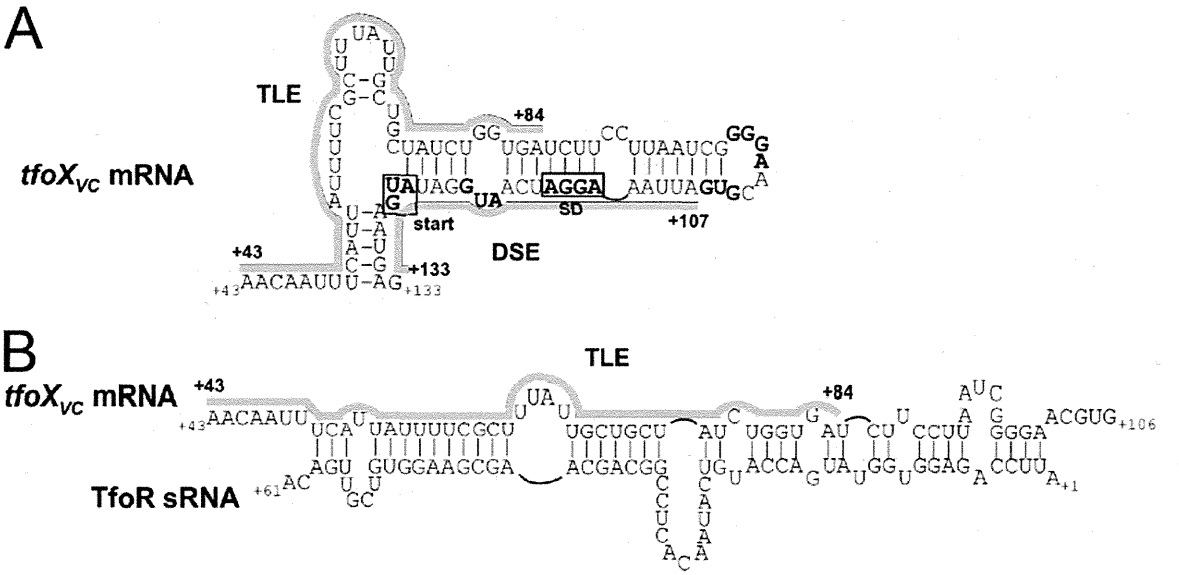
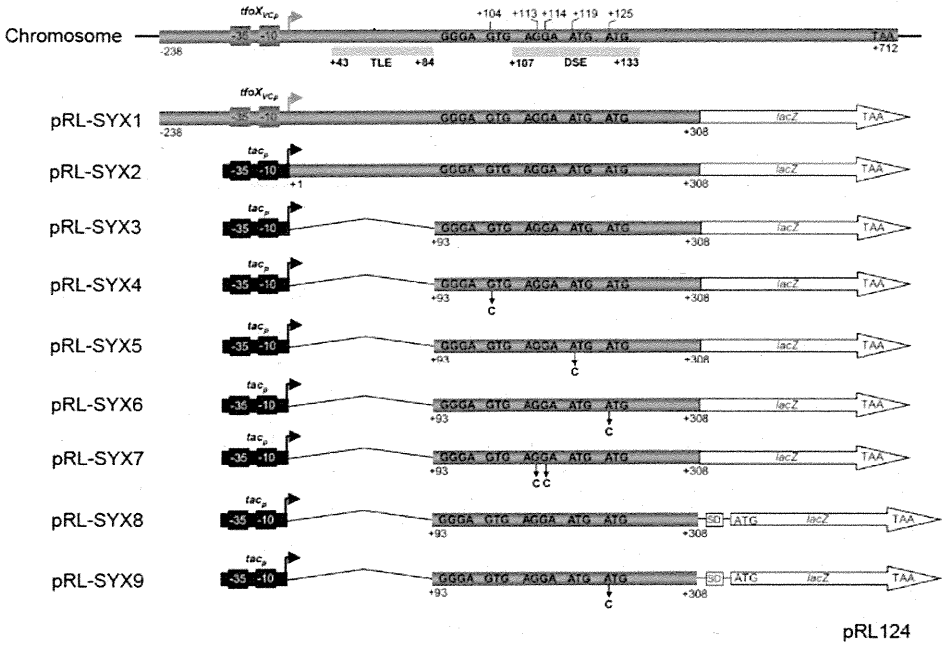


FIG. 1. (A) Mfold prediction of secondary structure of $tfoX_{VC}$ mRNA (71). Positions corresponding to TLE and DSE are drawn in gray. Possible base pairs are indicated by bars. Putative start codon and SD sequence for $tfoX_{VC}$ are shown by bold letters. Of these, confirmed sequences were enclosed. $\Delta G = -16.13$ kcal/mol. (B) Prediction of a base pairing between $tfoX_{VC}$ (upper) and TfoR (lower) RNAs using RNAhybrid. $\Delta G = -43.3$ kcal/mol.

reduced nearly to a background level under both culture conditions (Fig. 2). We also changed the A_{+125} to C in the tac_P/ΔTLE-based transcriptional fusion (pRL-SYX8) to generate pRL-SYX9 (Fig. 2). The two strains carrying pRL-SYX8 and pRL-SYX9 showed similar levels of expression (Fig. 2). These results indicated that the A_{+125}-to-C mutation impaired translation but not transcription. Thus, it appears plausible that $ATG_{+125\ to\ +127}$ acts as the start codon, rather than $GTG_{+104\ to\ +106}$ (Fig. 1A). According to the position of the start codon, the SD sequence could be comprised of $AGGA_{+112\ to\ +115}$ (Fig. 1A). Consistent with this, the base change of $AGGA_{+112\ to\ +115}$ to ACCA (pRL-SYX7) decreased β-galactosidase activity (Fig. 2). This notion is further supported by a previous report in which optimal spacing between the SD sequence and the start codon is described as about 8 to 10 nucleotides (nt) (7).

Genetic screening for the positive regulator of $tfoX_{VC}$. We previously predicted an intramolecular base pairing between the TLE and DSE RNAs using the Mfold software program (71, 76). When this structure was reevaluated using the information as described above (Fig. 2), TLE seemed to sequester the SD sequence within DSE (Fig. 1A). Based on these observations, we proposed a model for the translational control mechanism as follows. In the absence of $(GlcNAc)_2$, the TLE of the $tfoX_{VC}$ mRNA represses translation through base pairing with the DSE of the mRNA. On the other hand, in the presence of $(GlcNAc)_2$, the formation of this structure is somehow inhibited by an unknown substance, allowing the ribosome to bind to the site of DSE, which ultimately enhances translation. If this model is true, some regulatory factor(s) may be needed to activate translation of $tfoX_{VC}$ in response to $(GlcNAc)_2$. Therefore, we thought that it might be possible to clone the regulatory gene(s) by screening for increased translational activity of $tfoX_{VC}$. To achieve this, we constructed an *E. coli* strain, DH1 (20), with a $tfoX_{VC}$::*lacZ* translational fusion (SYEC001S) whose transcription was constitutively driven by the *bla* promoter from Tn*3* (14). SYEC001S was transformed with a plasmid library consisting of sonicated DNA fragments from a *V. cholerae* strain, V060002, cloned into the HincII site of pUC118. The resultant colonies were screened on LB plates containing X-Gal and Ap. On these plates, the starting strain appeared white (Lac^-). Among 20,000 colonies screened, a single blue colony (Lac^+) was isolated, which carried the plasmid pUC-SYR1. Retransformation of SYEC001S with pUC-SYR1 confirmed that this phenotype was a plasmid-linked trait. pUC-SYR1 increased β-galactosidase activity from the $tfoX_{VC}$::*lacZ* fusion in SYEC001S more than 30-fold (145 ± 18 Miller units [mean ± standard deviation] in LB) compared with that of pUC118 (4 ± 3 Miller units). These results suggest that pUC-SYR1 carries a positively *trans*-acting regulatory element of $tfoX_{VC}$. Sequence analysis showed that pUC-SYR1 contained the 332-bp intergenic region between the *VC2078* and *VC2079* genes (corresponding to nucleotide positions 2236523 to 2236854 on chromosome I in the *V. cholerae* strain N16961) (Fig. 3A). Computer analysis of the cloned fragment using GENETIX predicted no significant open reading frames. Instead, this fragment contained an apparent Rho-independent terminator oriented in the 3′ direction of the *lac* promoter and was already predicted to encode a 96-nt RNA (corresponding to nucleotide positions 2236607 to 2236702 on chromosome I in N16961) by an RNA deep-sequencing-based screen (33). These observations suggest that pUC-SYR1 might express a noncoding regulatory RNA. We called this gene in the 332-bp region "*tfoR*."

Effect of *tfoR* on $tfoX_{VC}$ expression in *V. cholerae*. To examine the effect of *tfoR* on $tfoX_{VC}$ expression in *V. cholerae*, we introduced a deletion mutation of *tfoR* into the strain V060002 with a $tfoX_{VC}$::*lacZ* transcriptional or translational fusion and assayed β-galactosidase activities from these strains grown in the presence of GlcNAc or $(GlcNAc)_2$ (Table 2). As de-



	β-Galactosidase activity (Miller units ± SD) in M9 minimal medium plus:	
	5 mM GlcNAc	5 mM $(GlcNAc)_2$
pRL-SYX1	35 ± 7	530 ± 18 (15)
pRL-SYX2	171 ± 12	3596 ± 45 (21)
pRL-SYX3	3997 ± 292	4311 ± 253 (1.1)
pRL-SYX4	3733 ± 83	3716 ± 320 (1.0)
pRL-SYX5	5020 ± 169	4288 ± 82 (0.9)
pRL-SYX6	63 ± 14	95 ± 11 (1.5)
pRL-SYX7	78 ± 10	85 ± 10 (1.1)
pRL-SYX8	7811 ± 154	8133 ± 82 (1.0)
pRL-SYX9	7399 ± 250	8160 ± 164 (1.1)
pRL124	25 ± 4	23 ± 7 (0.9)

FIG. 2. Determination of the translational initiation region of *tfoX*$_{VC}$. Left: the structures of the *tfoX*$_{VC}$::*lacZ* fusions on pRL124. Names of plasmids are indicated on the left. The upstream and coding regions of *tfoX*$_{VC}$ are shown in gray. *tfoX*$_{VCp}$ and *tac*$_p$ indicate the *tfoX*$_{VC}$ and *tac* promoters, respectively. A hooked arrow indicates the transcriptional start site and direction, and the transcriptional start site is numbered +1. Positions of putative start codons and SD sequences are indicated. Nucleotides changed by site-directed mutagenesis are shown below. Right: the expression of the *tfoX*$_{VC}$::*lacZ* fusion genes under the culture condition in the presence of GlcNAc or $(GlcNAc)_2$. β-Galactosidase activity units are presented as means and standard deviations (SD). Experiments were performed three times. Numbers in parentheses in the $(GlcNAc)_2$ columns show the relative difference from the culture containing only GlcNAc. The host strain used was SY0603S.

scribed previously (71), in the *tfoR*$^+$ background, the addition of $(GlcNAc)_2$ moderately (2-fold) activated the transcriptional fusion gene but strongly (21-fold) activated the translational fusion gene compared with that of GlcNAc (Table 2). However, in the Δ*tfoR* background, the expression of the translational fusion was scarcely increased by $(GlcNAc)_2$ (Table 2). Deletion of *tfoR* had no significant effect on the expression of the transcriptional fusion (Table 2). In addition, pUC-SYR1 stimulated the expression of the translational fusion (10- to 14-fold) independently of $(GlcNAc)_2$ compared with pUC118 (Table 2, compare *tfoX*$_{VC\text{-}TL}$::*lacZ*Δ*tfoR*/pUC118 and *tfoX*$_{VC\text{-}TL}$::*lacZ*Δ*tfoR*/pUC-SYR1). These results suggest that *tfoR* positively affects *tfoX*$_{VC}$ expression, mainly at the translational level, and multicopy *tfoR* bypasses the need of $(GlcNAc)_2$ for translational regulation.

Effect of *tfoR* on natural competence. The data presented above revealed *tfoR*-dependent translational activation of *tfoX*$_{VC}$, encoding a positive regulator of natural competence. Therefore, we tested the effect of *tfoR* on transformation. After the competence-inducing treatment, the wild-type strain was transformed with a frequency of 1.3×10^{-8} (Table 3). However, the *tfoR* mutant strain was not transformed under the same culture conditions (Table 3). The introduction of pUC-SYR1 carrying the *tfoR*$^+$ gene into the *tfoR* mutant induced transformation regardless of the presence or absence of $(GlcNAc)_2$, confirming that *tfoR* was required for competence. Furthermore, pUC-SYR1 did not stimulate transformation in the *tfoX*$_{VC}$ mutant (Table 3). This indicates that the positive effect of *tfoR* on competence is mediated through *tfoX*$_{VC}$. It was noted that the *tfoR* mutant strain with pUC-SYR1 had a much higher efficiency of transformation (68- to 330-fold) than the wild-type strain (Table 3), although translation activity of *tfoX*$_{VC}$ in the strain with pUC-SYR1 was slightly lower (2- to 2.5-fold; for example, compare the activities of 317 ± 10 units and 129 ± 5 units in Table 2) than the activity from the wild-type strain grown in the presence of $(GlcNAc)_2$ (Table 2). This discrepancy is discussed in the following section.

Identification of a novel RNA activator. To detect RNA product from *tfoR*, we performed Northern blotting by using a *tfoR*-specific RNA probe. This probe was created to detect the transcript that ended at the site of a putative Rho-independent terminator. Total RNA was isolated from cells grown under the same culture conditions for β-galactosidase assays. In the presence of $(GlcNAc)_2$, an ~100-nt RNA was detected from the total RNA prepared from the wild-type cells, whereas no bands were observed in the total RNA from the *tfoR* mutant cells (Fig. 4). We could also detect this sRNA in the *tfoX*$_{VC}$ mutant (Fig. 4). Furthermore, when the RNA probe for the opposite transcript was used, the sRNA was not detected (data not shown). The *tfoR* mutant strain with pUC-SYR1 produced independently of $(GlcNAc)_2$ much larger amounts of the 100-nt RNA than the wild-type strain (Fig. 4), which correlated with a remarkable increase in transformation efficiency (Table 3). However, the introduction of pUC-SYR1 into the *tfoR*

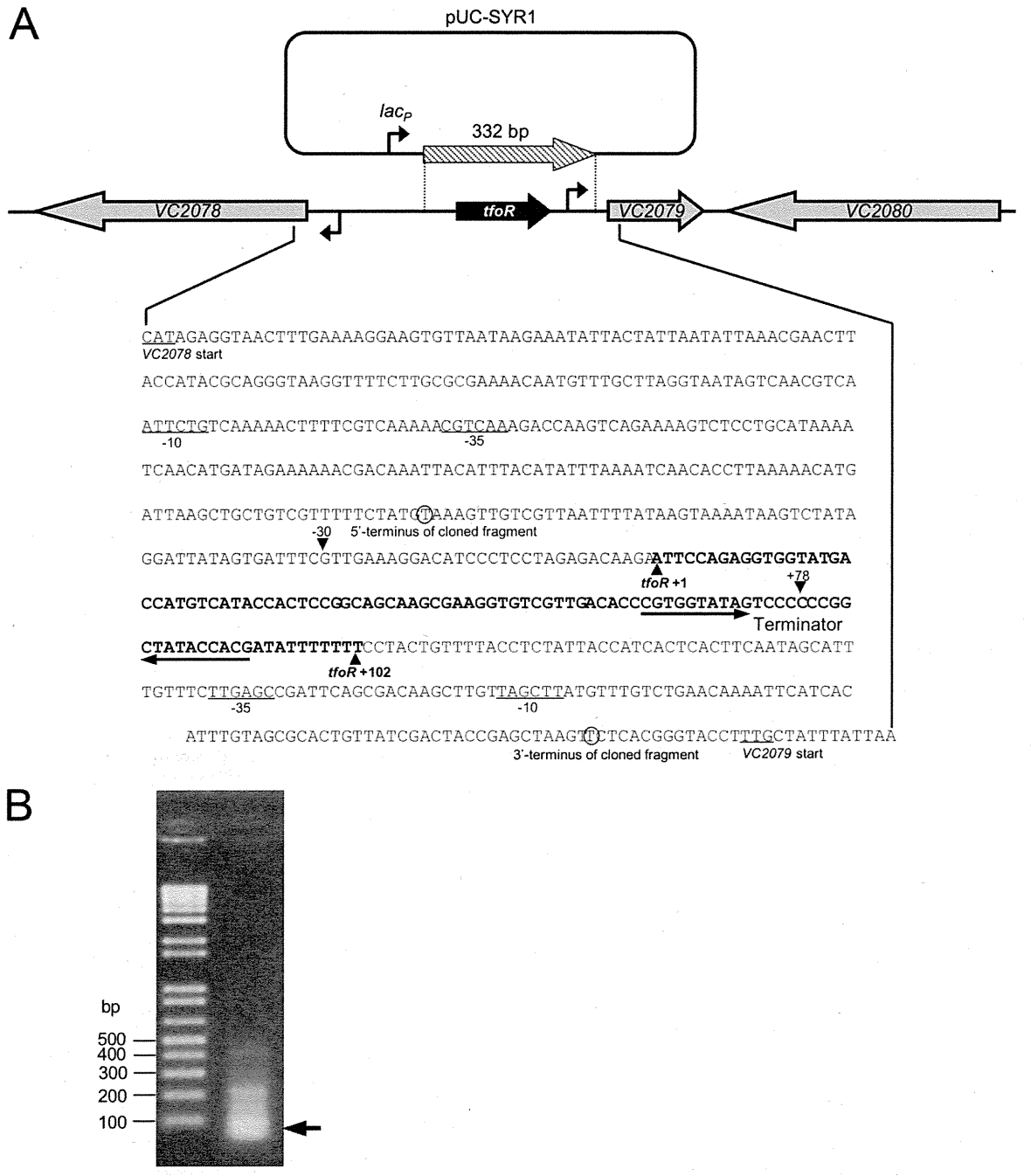


FIG. 3. Identification of the *tfoR* gene. (A) Intergenic region between the *VC2078* and *VC2079* genes. The cloned fragment in pUC-SYR1 is indicated by the shaded arrow that points in same direction as the *lac* promoter. The *tfoR* gene revealed by circularization RT-PCR is shown by bold letters. The *lacZ* reporter gene was inserted into the position immediately downstream of nucleotide −30 or +78 (see Table 4). (B) Products amplified by circularization RT-PCR. The major products indicated by an arrow were cloned into pGEM-T and transformed in *E. coli*, and 7 clones from independent transformants were sequenced.

mutant was not much more effective for $tfoX_{VC}$ expression than the addition of $(GlcNAc)_2$ to the wild-type strain (Table 2). The difference between a multicopy effect of *tfoR* on $tfoX_{VC}$ and on competence may suggest an additional positive role of *tfoR* in competence beyond the $tfoX_{VC}$ regulation. Additionally, we detected several longer RNAs (200 to 500 nt), as well as the 100-nt RNA in the strain carrying pUC-SYR1 (Fig. 4). As expected from their sizes, the longer species may be hybrid transcripts that contain both sequences of *tfoR* and pUC118.

The 5′ and 3′ ends of the $(GlcNAc)_2$-induced sRNA were determined by circularization RT-PCR (11, 73). Briefly, the 3′ and 5′ ends of a linear RNA molecule were joined by T4 RNA ligase, and a region containing the 3′-5′ junction was converted to cDNA by reverse transcriptase. The cDNA was successively

TABLE 2. Effect of *tfoR* on expression of $tfoX_{VC}$::*lacZ* fusions in *V. cholerae*

V. cholerae lac fusion[a] or mutation	Plasmid	β-Galactosidase activity[b] (Miller units ± SD) in M9 minimal medium plus:		Fold activation[c]
		5 mM GlcNAc	5 mM $(GlcNAc)_2$	
$tfoX_{VC\text{-}TL}$::*lacZ*		15 ± 3	317 ± 10	21
$tfoX_{VC\text{-}TL}$::*lacZ* Δ*tfoR*		13 ± 3	9 ± 2	0.7
$tfoX_{VC\text{-}TL}$::*lacZ* Δ*tfoR*	pUC118	15 ± 2	8 ± 3	0.5
$tfoX_{VC\text{-}TL}$::*lacZ* Δ*tfoR*	pUC-SYR1	157 ± 15	129 ± 5	0.8
$tfoX_{VC\text{-}TC}$::*lacZ*		157 ± 5	320 ± 20	2.0
$tfoX_{VC\text{-}TC}$::*lacZ* Δ*tfoR*		139 ± 10	250 ± 15	1.8

[a] TL and TC indicate translational fusion and transcriptional fusion, respectively. Strains used are as follows: $tfoX_{VC\text{-}TL}$::*lacZ*, SY0616S; $tfoX_{VC\text{-}TL}$::*lacZ* Δ*tfoR*, SY0616S26K; $tfoX_{VC\text{-}TC}$::*lacZ*, SY0613S; $tfoX_{VC\text{-}TC}$::*lacZ* Δ*tfoR*, SY0613S26K.

[b] β-Galactosidase activity units are presented as means ± standard deviations. Experiments were performed three times.

[c] Fold activation is indicated as the relative difference from the value for the culture containing only GlcNAc.

amplified by PCR, with one primer close to the 3′ end and the other to the 5′ end. Cloning and sequencing of amplified DNA fragments would definitively demonstrate the number and species of nucleotides between the 3′ and 5′ ends of a given transcript. After ligation, total RNA was subjected to reverse transcription and synthesized cDNA was utilized as a template for PCR. The major band (slightly less than 100 bp) of PCR products (Fig. 3B) was cloned into pGEM-T and transformed in *E. coli*, and 7 clones from independent transformants were sequenced. All clones exhibited the same 5′ end, "A" (Fig. 3A). This established that the 5′ end of the sRNA was the A nucleotide indicated as +1, which was located 1 nt downstream of the 5′ end of the sRNA previously predicted by Liu et al. (33). From the sequence result of 6 among 7 analyzed clones, it was indicated that the 3′ end of sRNA was the last "U" of 7 U's following the terminator stem (Fig. 3A), which was suggested to be the major transcription termination site. Based on these results, we estimated the sRNA to be 102 nt long. This is consistent with the size of the sRNA obtained from Northern blotting (Fig. 4).

TABLE 3. Effect of *tfoR* on natural transformation

Description of *V. cholerae* donor strain[a]	Plasmid	Transformation efficiency (Cm^r CFU/total CFU)[b] in M9 minimal medium plus:	
		5 mM GlcNAc	5 mM $(GlcNAc)_2$
Wild type		<DL[c]	1.3×10^{-8}
Δ*tfoR*		<DL	<DL
Δ*tfoR*	pUC118	<DL	<DL
Δ*tfoR*	pUC-SYR1	8.8×10^{-7}	4.3×10^{-6}
$\Delta tfoX_{VC}$	pUC118	<DL	<DL
$\Delta tfoX_{VC}$	pUC-SYR1	<DL	<DL

[a] Strains used are as follows: wild type, V060002; Δ*tfoR*, SY0626K; $\Delta tfoX_{VC}$, SY0608S.

[b] Representative data from at least two independent transformations are shown. Transformation efficiencies of strains carrying plasmid were defined as Cm- and Ap-resistant CFU divided by Ap-resistant CFU.

[c] <DL, Below detection limit, ~1.0×10^{-9}.

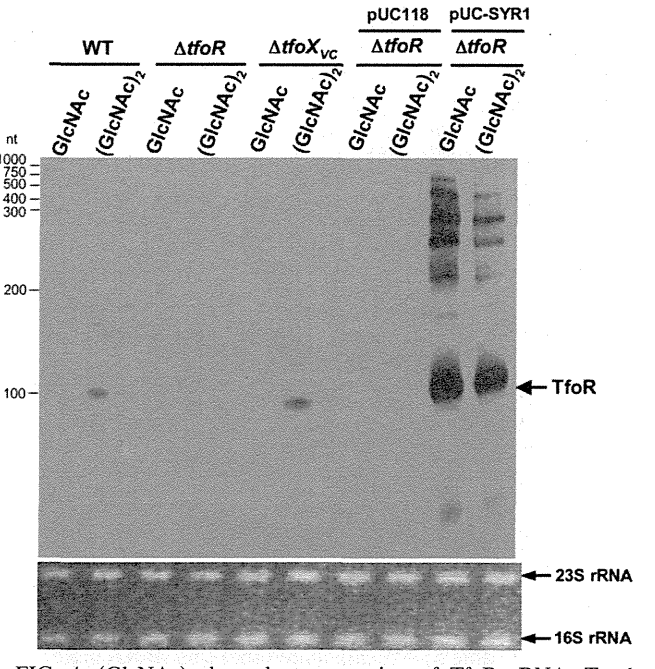


FIG. 4. $(GlcNAc)_2$-dependent expression of TfoR sRNA. Total RNA was isolated from cells grown in the presence of GlcNAc or $(GlcNAc)_2$, separated on a polyacrylamide gel containing 6 M urea, and transferred onto a nylon membrane. RNA product from *tfoR* was detected by Northern blotting. The lower panel indicates the ethidium bromide-stained total RNA pattern. Strains used were the WT (wild type), V060002; the Δ*tfoR* strain, SY0626K; and the $\Delta tfoX_{VC}$ strain, SY0608S.

We could not find a significant promoter immediately upstream of the 5′ end of the sRNA. To evaluate the transcriptional response of the sRNA gene to $(GlcNAc)_2$, we fused the *lacZ* gene to a site within the terminator sequence (immediately downstream of nucleotide +78) (Fig. 3A). The expression of the fusion gene was induced 16-fold by $(GlcNAc)_2$ (Table 4). Moreover, we inserted *lacZ* into a further upstream nucleotide (immediately downstream of nucleotide −30) (Fig. 3A). The fusion gene expression still responded to $(GlcNAc)_2$ (Table 4), suggesting that the $(GlcNAc)_2$-responsive promoter may be located further upstream of the −30 nucleotide G.

We cloned this sRNA gene (nucleotides +1 to +102) in pBAD33. The expression of the RNA was inducible under the control of the *araBAD* promoter. This plasmid, pBAD-SYR1,

TABLE 4. Analysis of *tfoR*::*lacZ* fusion expression in *V. cholerae*

tfoR::*lacZ* fusion[a]	β-Galactosidase activity[b] (Miller units ± SD) in M9 minimal medium plus:		Fold activation[c]
	5 mM GlcNAc	5 mM $(GlcNAc)_2$	
$tfoR_{+78}$::*lacZ*	7 ± 3	109 ± 9	16
$tfoR_{-30}$::*lacZ*	7 ± 1	164 ± 5	23

[a] Strains used are as follows: $tfoR_{+78}$::*lacZ*, SY0627; $tfoR_{-30}$::*lacZ*, SY0628.

[b] β-Galactosidase activity units are presented as means ± standard deviations. Experiments were performed three times.

[c] Fold activation is indicated as the relative difference from the value for the culture containing only GlcNAc.

TABLE 5. Effect of *tfoR* and *hfq* on expression of the *tfoX*$_{VC}$::*lacZ* translational fusion in *E. coli*

Strain description[a]	Plasmid(s)	β-Galactosidase activity[b] (Miller units ± SD) in LB plus:			
		Nothing	Arabinose	IPTG	Arabinose + IPTG
Wild type	pBAD33	7 ± 3	7 ± 1	ND[c]	ND
	pBAD-SYR1	15 ± 1	201 ± 12	ND	ND
	pBAD33 + pTrc99A	8 ± 1	8 ± 1	20 ± 1	13 ± 1
	pBAD-SYR1 + pTrc99A	17 ± 1	172 ± 18	21 ± 1	183 ± 13
Δ*hfq*	pBAD33 + pTrc99A	11 ± 1	6 ± 1	9 ± 2	10 ± 3
	pBAD-SYR1 + pTrc99A	12 ± 1	11 ± 2	9 ± 2	9 ± 2
	pBAD33 + pTrc-hfq	16 ± 3	10 ± 1	13 ± 2	17 ± 3
	pBAD33 + pTrc-SYQ1	12 ± 1	11 ± 1	15 ± 3	14 ± 2
	pBAD-SYR1 + pTrc-hfq	12 ± 2	51 ± 8	17 ± 4	215 ± 19
	pBAD-SYR1 + pTrc-SYQ1	14 ± 1	83 ± 16	20 ± 2	231 ± 10

[a] Strains used are as follows: Wild type, SYEC001S; Δ*hfq*, SYEC001SQ1.
[b] β-Galactosidase activity units are presented as means ± standard deviations. Experiments were performed three times.
[c] ND, not done.

was used to transform SYEC001S, an *E. coli* strain with a *tfoX*$_{VC}$::*lacZ* translational fusion gene. Induction of transcription of the sRNA gene in this plasmid was necessary and sufficient to stimulate the expression of the *tfoX*$_{VC}$::*lacZ* translational fusion (Table 5). We concluded that this sRNA gene is *tfoR*, and we called this novel sRNA "TfoR."

Effect of Hfq on TfoR-mediated regulation of *tfoX*$_{VC}$. The majority of bacterial sRNA regulators act through base pairing with RNAs, resulting in the modulation of translation and stability of mRNAs (68). The RNAhybrid program (26) was used to search for a possible complementary sequence in the 5′ untranslated region (UTR) of *tfoX*$_{VC}$ mRNA with the sequence of TfoR. TfoR was found to extensively match with the region including TLE of *tfoX*$_{VC}$ mRNA (Fig. 1B), which acts negatively on translation (71). Therefore, TfoR might contribute to the activation of translation through intermolecular base pairing with TLE. In many cases, the RNA chaperone Hfq is required for sRNA-mediated regulation to affect the processing or stability of the sRNA transcript (40, 46, 75) or to facilitate RNA-RNA interactions of sRNA and target mRNA (46, 74). To examine whether Hfq affects the translational regulation of *tfoX*$_{VC}$ by TfoR, we introduced a deletion mutation of *hfq* into the V060002 background. However, the *hfq* mutation severely impaired growth in M9 minimal medium containing $(GlcNAc)_2$ (data not shown). For such a methodological limitation in *V. cholerae*, we reconstituted an experimental system based on the *E. coli* reporter strain SYEC001S to analyze the Hfq effect. We first deleted the *hfq* gene in SYEC001S to generate strain SYEC001SQ1, and then *tfoX*$_{VC}$::*lacZ* expression was analyzed when TfoR and Hfq were expressed under the control of the *araBAD* promoter of pBAD33 and the *tac* promoter of pTrc99A, respectively. In the *hfq* mutant, either induction of TfoR from pBAD-SYR1 or Hfq from pTrc-hfq and pTrc-SYQ1 (carrying the *E. coli hfq* gene and the *V. cholerae hfq* gene, respectively) failed to stimulate translation, while both induction of TfoR and of Hfq restored the capability for activating translation (Table 5). Northern blotting showed that TfoR was detectable only from the *hfq*$^+$ strains (Fig. 5), suggesting that Hfq may be required for TfoR expression.

TfoR activates translation from *tfoX*$_{VC}$ mRNA *in vitro*. For *in vitro* translation of *tfoX*$_{VC}$, we used a reconstituted PURE system that includes only purified ribosomes and all other translation factors (54). This system is successfully used elsewhere for *in vitro* translation analysis (38, 48, 66). We *in vitro* synthesized *tfoX*$_{VC}$::*flag* mRNA (full length: nucleotides +1 to +709 translationally fused to the FLAG tag sequence) as a template and TfoR sRNA. An RNA complementary to TfoR was also synthesized as a negative control. The TfoX$_{VC}$::FLAG protein was analyzed by Western blotting with anti-FLAG antibody. TfoX$_{VC}$::FLAG was synthesized in the presence of TfoR at an equimolar concentration of the template RNA, whereas the addition of the RNA complementary to TfoR at up to a 10-fold molar excess over the RNA template did not stimulate TfoX$_{VC}$::FLAG synthesis (Fig. 6). This indicates that TfoR is required specifically for translational activation of *tfoX*$_{VC}$. We also synthesized *tfoX*$_{VC}$::*flag* mRNA with deletion of TLE (ΔTLE: nucleotides +93 to +709). The deletion made the translation constitutively active *in vivo* (71). Consistently, the TfoX$_{VC}$::FLAG protein was synthesized from the ΔTLE mRNA even in the absence of TfoR, which was not further activated by the addition of TfoR (Fig. 6). This suggests that the TLE of the *tfoX*$_{VC}$ mRNA could be a regulatory target of TfoR.

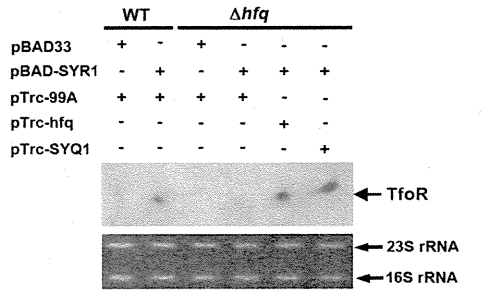


FIG. 5. Effect of *hfq* on TfoR expression in *E. coli*. Total RNA was isolated from cells carrying pBAD33 (or pBAD-SYR1) and pTrc99A (or pTrc-hfq/pTrc-SYQ1) grown in LB containing Cm, Ap, arabinose, and IPTG, separated on a polyacrylamide gel containing 6 M urea, and transferred onto a nylon membrane. The TfoR sRNA was detected by Northern blotting. The lower panel indicates the ethidium bromide-stained total RNA pattern. Strains used were the WT (wild type), SYEC001S, and the Δ*hfq* strain, SYEC001SQ1.

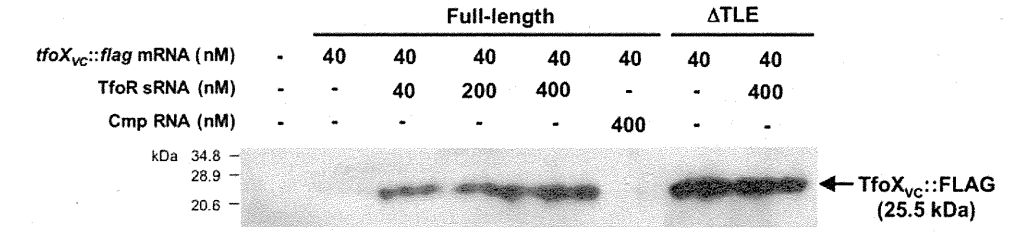
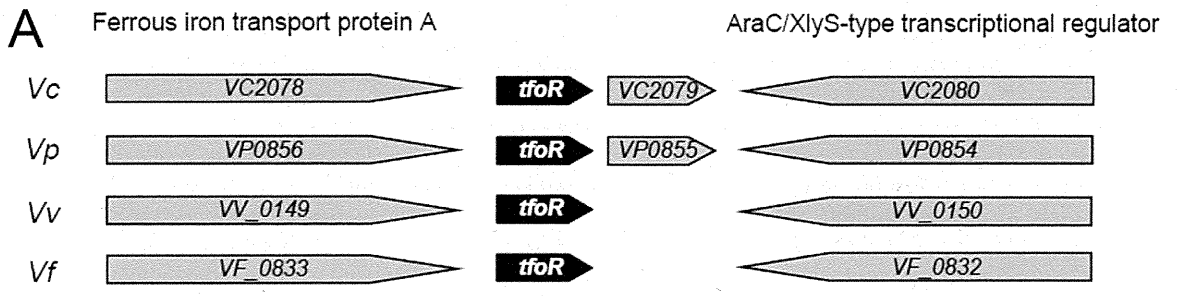


FIG. 6. Effect of TfoR on translation from $tfoX_{VC}$ mRNA *in vitro*. Template $tfoX_{VC}$::*flag* mRNAs (full-length, nucleotides +1 to +709, and ΔTLE, nucleotides +93 to +709, both of which are translationally fused to the FLAG tag sequence) and TfoR sRNA were synthesized *in vitro*. An RNA complementary to TfoR (Cmp RNA) was also synthesized to be used as a negative control. Each $tfoX_{VC}$::*flag* mRNA was mixed with indicated concentrations of TfoR sRNA. Protein synthesis was carried out in a PURE system. The synthesized $TfoX_{VC}$::FLAG protein was separated by SDS-PAGE and analyzed by Western blotting with anti-FLAG antibody.

TfoR is conserved in *Vibrio* spp. In addition to *V. cholerae*, several *Vibrio* spp., including *V. vulnificus*, *V. fischeri*, and *V. parahaemolyticus*, show natural competence in the presence of chitin (8, 17, 50). These and many other *Vibrio* spp. have not only the *tfoX* gene but also DNA uptake genes (31, 50), suggesting a conservation of the TfoX regulon for competence induction. Using the BLAST program (1), we found homologues of *V. cholerae tfoR* ($tfoR_{VC}$) in many sequenced genomes of the *Vibrio* spp. Of these, Fig. 7 shows the sequences homologous to that of $tfoR_{VC}$ from known competent species as above. In every case, the *tfoR* locus was situated between a gene encoding ferrous iron transport protein A (VC2078, VP0856, VV1_0149, and VF_0833) and a gene encoding a hypothetical protein homologous to an AraC/XylS-type transcriptional regulator (VC2080, VP0854, VV1_0150, and VF_0832) (Fig. 7A). These genomic features may indicate functional relatedness within the gene cluster. Multiple alignment analysis (62) showed a high sim-



B

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Vc tfoR    TCGTTAATTTTATAAGTAAAATAAGTCTATAGGATTATAGTGA-TTTCGTTGAAAGGACA
Vp tfoR    TCGTTATTTTTATAAGAAAAATAAGTAAGGTCTCGTATAGTTA-TTCCATCGAGAGGGCA
Vv tfoR    GCGTTATTTTTATAAGGAAAATAAGTCGATTAACTTATAGTGA-ACACATCGAGAGG---
Vf tfoR    TCGTTATTTTGATATGTAAAAAAAGAAGAGGCAGTTATAGTTAATTACATCGAGAGGGAG
           .******.***.***.* ****.***.  .      .****** * .. *.*.**.***

                          +1
Vc tfoR    TCCCTCCTAGAGACAAGAATTCCAGAGGTGGTATGACCATGTCATACCACTCCGGCAGCA
Vp tfoR    AC-CTCTTA----AAAGAATTCCAGAGGTGGCATGGCAA---CATGTCACTCCGGCAGCA
Vv tfoR    -CACTCCTCT---CAAGAATTCCAGAGGTGACGTGACGATGTCGCGTCACTCCGGCAGCA
Vf tfoR    AC-CTCTCAACTTTTATTCTAATAGAAGTAACAAGAAG-TATCTTGTTACTTCGGCAGCA
            * *** ..     .*...*...***.**. ...*.. .. .* ....***.********

                                  terminator
Vc tfoR    AGCGAAGGTGTCGTTGACACCCGTGGTATAGTCCCCCCGGCTATACCACGATATTTTTTT
Vp tfoR    AGCGAAGGTGTCATTGACACCCGTGGTATAGTCCCCCCGGCTATACCACGATATTTTTTT
Vv tfoR    AGCGAAGGTGTCATTGACACCCGTGGTATAGTCCCCCCGGCTATACCACGATATTTTTTT
Vf tfoR    AGCGAAGGCGTCTTTGGCGCCCGTGGCGTAGTCCCCCCGGCTACGTCACGATCTTTTTTT
           ********.*** ***.*.*******..***************...*******.*******
```

FIG. 7. Conservation of TfoR in the *Vibrio* spp. (A) The genomic location of the *tfoR* gene. Bacterial strains shown are as follows: *Vc*, *V. cholerae* N16961; *Vp*, *V. parahaemolyticus* 2210633; *Vv*, *V. vulnificus* CMCP6; *Vf*, *V. fischeri* ES114. In the genome sequences of *V. vulnificus* and *V. fischeri*, the gene corresponding to *VC2079* or *VP0855* is not annotated. (B) Alignment of *tfoR* sequences in the 4 species. Asterisk and dot symbols show sequence identity and similarity, respectively. "+1" indicates the 5′ terminus of TfoR in *V. cholerae*. The putative −10 sequences similar to the σ^{70}-type promoter are underlined.

ilarity of *tfoR* (Fig. 7B). Furthermore, using the RNAhybrid program, we could predict significant base pairing of *tfoR* and *tfoX* in each species (data not shown). The presence of both *tfoR* and *tfoX* in various *Vibrio* spp. suggests the potential for a conserved mechanism of competence regulation across this genus.

DISCUSSION

TfoX is the central regulator of DNA uptake by the naturally competent bacteria *H. influenzae* and *V. cholerae*. Under competence-inducing conditions, *tfoX* gene expression is activated at the transcriptional and translational levels in both organisms (6, 71), but their precise regulatory mechanisms have remained unknown. We have investigated the mechanism of $tfoX_{VC}$ translational control in *V. cholerae*, which is more prominent than the transcriptional control. In this study, we identified a novel sRNA, TfoR sRNA, by a random screening of clones that are able to stimulate $tfoX_{VC}$::*lacZ* translation (Fig. 3; see also "Genetic screening for the positive regulator of $tfoX_{VC}$" above). We showed that TfoR activated $tfoX_{VC}$ translation both *in vivo* and *in vitro* (Tables 2 and 5 and Fig. 6) and was required for natural competence (Table 3). In addition, transcription of *tfoR* was induced by $(GlcNAc)_2$ (Table 4 and Fig. 4). On the basis of these results, we propose that in the presence of $(GlcNAc)_2$, TfoR sRNA is expressed to activate the translation of $tfoX_{VC}$, which leads to the induction of natural competence. This is the first report of the identification of a posttranscriptional regulator for the expression of the *tfoX* gene. Regarding translational control in these *H. influenzae* and *V. cholerae* bacteria, however, the mechanisms are likely to be different, because there is no considerable sequence similarity between the 5′ UTRs of $tfoX_{VC}$ and $tfoX_{HI}$ (data not shown), and *H. influenzae* has no homologue of TfoR. On the other hand, *Vibrio* spp. seem to share the *tfoR* gene (Fig. 7) as well as the *tfoX* gene (50). This suggests that the *tfoR*/*tfoX* regulatory system may be important for controlling natural competence in *Vibrio* spp.

Many bacterial sRNA regulators act through base pairing with RNAs, resulting in the modulation of translation and stability of mRNAs (68). They can often base pair at the SD sequence and thus block translation initiation by preventing ribosome binding (68). In addition to repressing translation, sRNAs can also activate translation, in many cases by preventing the formation of an intrinsic structure inhibitory to translation initiation (19, 37, 47, 51, 66), which is called an "anti-antisense mechanism" (16, 37). Using the RNAhybrid program, TfoR was predicted to extensively pair with the region including TLE of $tfoX_{VC}$ mRNA (Fig. 1), which acts negatively on translation (71). Therefore, TfoR might act as an antiantisense RNA through intermolecular base pairing with TLE to counteract its translational inhibitory effect. In many cases, sRNAs require the RNA chaperone Hfq for processing or stability (40, 46, 75) and for productive base pairing with target mRNAs (46, 74). In our *V. cholerae* strain background, disruption of *hfq* showed a phenotype with slow growth in M9 minimal medium containing $(GlcNAc)_2$, suggesting that this mutation might affect utilization of $(GlcNAc)_2$. Instead, our *in vivo* analysis in *E. coli* revealed the following: (i) that both Hfq and TfoR are required for activating translation of $tfoX_{VC}$ (Table 5), and (ii) that TfoR expression is remarkably decreased in the absence of Hfq (Fig. 5). On the other hand, TfoR could activate translation *in vitro* (PURE system) without adding the Hfq protein (Fig. 6). In order to explain the conflicting results, one possibility suggested is that Hfq affects translation of $tfoX_{VC}$ *in vivo* through modulating stability of TfoR. However, we cannot rule out the additional possibility that Hfq directly facilitates interactions of TfoR and $tfoX_{VC}$ RNAs. Therefore, further *in vivo* and *in vitro* analyses will be necessary to understand the role of Hfq in the TfoR regulation.

We have yet to determine the mechanism underlying TfoR expression. We found that the transcription of *tfoR* was significantly induced by $(GlcNAc)_2$ (Table 4). Such a transcriptional response still occurred even though the *lacZ* reporter gene was fused immediately downstream of nucleotide −30 (Table 4). This suggests that a promoter that is directly or indirectly responsive to $(GlcNAc)_2$ might be located further upstream of the position −30. In the multiple alignment analysis in Fig. 7B, a conserved sequence (TATAGT) similar to the −10 box of the σ^{70} promoter could be found at the nucleotides −42 to −37. If this acted as the promoter, longer RNA (at least 130 nt) would be primarily transcribed from it and then posttranscriptionally processed to yield the mature TfoR sRNA (102 nt). In this study, however, we could not detect such a longer transcript (Fig. 4). More extensive analysis will be required to elucidate the mechanisms of TfoR expression.

sRNA regulators have been identified in a wide range of bacteria and found to play critical regulatory roles in many physiological processes, for example, replication (63), outer membrane protein synthesis (45), sugar transport (67), stress response (2), iron homeostasis (40), virulence (47), and quorum sensing (29). In *V. cholerae*, several sRNAs have been identified through genetic screens and computational methods (13, 29, 58). Additionally, direct cloning and parallel sequencing experiments identified numerous sRNAs with unknown functions in this bacterium (33). In many competent bacteria, the development of a competent state is tightly controlled depending on environmental changes (10). This may mean that bacteria limit the incidence of genetic exchange caused by DNA uptake under unfavorable conditions. In both transformable species, *Streptococcus pneumoniae* and *B. subtilis*, competence is induced by a small peptide pheromone (22, 36). A dedicated two-component regulatory system detects the pheromone and transmits a signal, which leads to activation of the key transcriptional regulator for the competence regulon (10, 28, 64). On the other hand, *H. influenzae* utilizes the cAMP-CRP system to activate transcription of the $tfoX_{HI}$ gene (6, 77). To our knowledge, the present study is the first example of the involvement of sRNA in competence regulation. sRNAs are faster to produce than proteins and act posttranscriptionally. Thus, they could shut off or turn on gene expression more rapidly than protein-based transcriptional regulators. It is probable that sRNA-mediated control of $tfoX_{VC}$ expression confers an advantage to *V. cholerae* cells in quickly adjusting DNA uptake according to environmental changes. $TfoX_{VC}$ acts not only as a central regulator of the competence regulon but also as a positive regulator of several genes for extracytoplasmic chitin degradation (41), and thus it could promote degrada-

tion of chitin into oligosaccharides, including $(GlcNAc)_2$, a major inducer of $tfoX_{VC}$ expression, generating a positive feedback loop. Therefore, it is reasonable to assume that TfoR may accelerate this regulatory loop via posttranscriptional activation of $tfoX_{VC}$.

In addition to $tfoX_{VC}$ (*VC1153*), *V. cholerae* has another *tfoX* paralogue, $tfoY_{VC}$ (*VC1722*) (50), which was originally named $tfoX_{GEMM}$ (69). Both orthologs of $tfoX_{VC}$ and $tfoY_{VC}$ are present in other *Vibrio* spp., *V. parahaemolyticus*, *V. vulnificus*, and *V. fischeri* (50). Interestingly, the 5′ UTR of $tfoY_{VC}$ mRNA is reported to function as a cyclic di-GMP-sensing riboswitch to posttranscriptionally control its expression (27, 56, 61). Disruption of $tfoY_{VC}$ had no significant effect on chitin-induced transformation (S. Yamamoto, unpublished data); however, this gene might be involved in competence induction under some favorable environmental conditions. If this is the case, *V. cholerae* (and probably other *Vibrio* spp.) utilizes different posttranscriptional control mechanisms of paralogous regulators to regulate competence. Such regulatory modes of $tfoX_{VC}$ and $tfoY_{VC}$ might allow this bacterium to respond rapidly to different conditions required for DNA uptake.

ACKNOWLEDGMENTS

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